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(54) Title: INHIBITION OF ONCOGENE TRANSCRIPTION BY SYNTHETIC POLYAMIDES

(57) Abstract

Polyamides capable of inhibiting the transcription of oncogenes are provided. Preferred polyamides comprising at least three complementary pairs of aromatic carboxamide residues bind in the minor groove of double-stranded DNA. With regard to each polyamide, the complementary pairing of some or all of the polyamide's aromatic carboxamide residues determines the target nucleotide sequence of an identified dsDNA target in the promoter region of an oncogene.

A

The HER-2/neu promoter

5'CCCGGGGGTCCTGGAAGCCACAAGGTAAACACAACACATCCCCCTCCTGGACTATGCAAT

TTTACTAGAGGATGTGGTGGGAAAACCATTATTTGATATTAAAACAAATAGGCTTGGGATGG

AGTAGGATGCAAGCTCCCCAGGAAAGTTTAAGATAAAACCTGAGACTTAAAAGGGTGTTAAG

AGTGGCAGCCTAGGGAATTTATCCCGGACTCCGGGGAGTCACCAGCCTCTGCATTTAGGGAT

AP-2

TCTCCGAGGAAAAGTGTGAGAACGGCTGCAGGCAACCCAGGCGTCCCGGCGCTAGGAGGGAC

GACCCAGGCCTGCGCGAAGAGAGGGAGAAAGTGAAGCTGGGAGTTGCCGACTCCCAGACTTC

GTTGGAATGCAGTTGGAGGGGGCGAGCTGGGAGCGCGCTTGCTCCCAATCACAGGAGAAGGA

ESX TATA

GGAGGTGGAGGAGGAGGGCTGCTTGAGGAAGTATAAGAATGAAGTTGTGAAGCTGAGATTCC

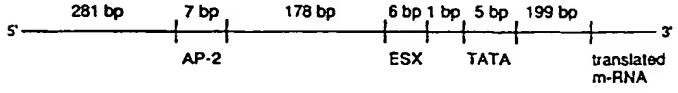
CCTCCATTGGGACCGGAGAAACCAGGGGAGCCCCCCGGGCAGCCGCGCGCCCCTTCCCACGG

GGCCCTTTACTGCGCCGCGCGCCCGGCCCCCACCCCTCGCAGCACCCCGCGCCCCGCGCCCT

CCCAGCCGGGTCCAGCCGGAGCCATGGGGCCGGAGCCGCAGTGAGCACCATG-3'

B

Schematic representation of the promoter



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INHIBITION OF ONCOGENE TRANSCRIPTION BY SYNTHETIC POLYAMIDES

The U.S. Government has certain rights in this invention pursuant to Grant Nos. GM 26453, 27681 and 47530 awarded by the National Institute of Health.

BACKGROUND OF THE INVENTION

Related Application

The patent application claims priority to U.S. provisional patent application 60/099,854, filed September 11, 1998, which is hereby incorporated by reference in its entirety.

Field of the Invention

This invention relates to specific polyamides that bind to predetermined nucleotide sequences in the minor groove of double-stranded DNA (dsDNA) located within the promoter region of a gene to prevent expression of endogenous oncogenes.

Description of the Related Art

The tyrosine kinase membrane growth factor receptor HER2/neu, also known as $p185^{HER2}$, is encoded by a cellular oncogene of the same name that is overexpressed and amplified in 20 to 30% of human breast cancers, among others, including human gynecologic adenocarcinomas, such as those of the ovary, endometrium, fallopian tube, and cervix. See Baert, J.-L. *et al.*, *Int. J. Cancer* **70**, 590-597 (1997); Benz, C., *et al.*, *Oncogene* **15**, 1513-1525 (1997); Chang, C.-H., *et al.*, *Oncogene* **14**, 1617-1622 (1997); Scott, G. K., *et al.*, *J. Biol. Chem.* **269**, 19848-19858 (1994); Pasleau, F.; *et al.*, *Oncogene* **8**, 849-854 (1993); Tal, M. *et al.*, *Molecular and Cellular Biology* 7, 2597-2601 (1987).

The neu oncogene gene product was originally described in chemically-induced (ethylnitosourea) tumors in rodents. Subsequently, the human counterpart, c-erbB-2 or Her-2/neu, was found to be homologous to the EGF receptor, a 185 kDa transmembrane

protein with protein tyrosine kinase activity. Overexpression of HER2/neu is also associated with the likelihood that tumors will metastasize, with a resulting poor prognosis for the patient. Mutation, amplification, and overexpression of the Her-2/neu oncogene has been reported to be associated with breast tumor progression, early metastasis and poor prognosis. Her-2/neu gene amplification directly correlates with lymph node metastasis. Additionally, in an animal model, activating mutations lead to rapid tumor progression. As a result, it is believed that the Her-2/neu protein likely plays a role in cell motility and hence in metastasis. Thus, inhibition of Her-2/neu gene expression by direct interference at the DNA level may be a potent therapeutic approach for metastatic disease.

Several transcription factors - such as ESX, AP-2, and TBP - play an important role in the regulation of the expression of this receptor. See Baert, *et al.*, *supra*; Benz, *et al.*, *supra*; Chang, *et al.*, *supra*; Bosher, J. M., *et al.*, *Proc. Natl. Acad. Sci. USA* **92**, 744-747 (1995). These transcription factors activate the expression of p185^{HER2} upon binding to sites within the HER2/neu promoter. The nucleotide sequence of the HER2/neu promoter and a schematic representation are shown in Figure 1. While the ESX binding site and the TBP binding site (TATA box) are close to each other, the OB2-1 (a member of the AP-2 transcription factor family) site is located about 180 base pairs further upstream.

ESX belongs to the *Ets* family of transcription factors. *Ets* proteins bind in a winged helix-turn-helix motif to the major groove of DNA contacting the minor groove on the flanking sides of the binding site. Karim, F. D., *et al.*, *Genes and Development* **1990**, 1451-1453 (1990); Donaldson, L. W., *et al.*, *EMBO Journal* **15**, 125-134 (1996); Graves, B., *et al.*, *Nature* **1996**, *384*, 322; Nye, J. A., *et al.*, *Genes and Development* **1992**, 975-990 (1992); Kodandapani, R., *et al.*, *Nature* **380**, 456-460 (1996).

In contrast, AP-2 binds as a dimer to the major groove of DNA; its DNA binding region is organized in a manner similar to that of a leucine zipper protein. Williams, T. & Tjian, R. *Science* **251**, 1067-1071 (1991). The structural element that is responsible for the DNA binding as well as for the protein/protein interaction (dimerization) is predicted to consist of a helix-span-helix motif. It is not clear if the span subunit that consists of about 80 amino acids contacts the minor groove of DNA.

TBP is a ubiquitous transcription factor that is involved in the activation of most protein-encoding genes. TBP is a DNA-binding protein that interacts with the minor groove of double-stranded DNA ("dsDNA"). It should be noted that, apart from ESX, AP-2, and TBP, there are other potential transcription factor binding sites within the HER2/neu and other oncogene promoters.

Considerable effort has been expended in the art to devise methods to interfere with gene expression in living cells in the hope that therapeutic strategies will come from these studies. These approaches include interference with the translation of messenger RNA into protein by the introduction of antisense oligonucleotides into cells (natural or peptide nucleic acid based) or by ribozyme-mediated destruction of specific RNAs. Several approaches for direct inhibition of gene transcription have also been attempted; these include triple helix forming oligonucleotides, designed or selected zinc finger peptides that recognize pre-determined sequences, and DNA-binding calicheamicin oligosaccharides.

For any therapeutic approach based in interference with gene expression to be successful, several criteria must be met by the therapeutic agent: first, the agent must not possess any general cell toxicity; second, the agent must be cell-permeable and, in the case of the DNA-binding agents, the compounds must transit to the nucleus and bind their target sequence with high affinity and specificity in the context of cellular chromatin; and, third, binding of the agent to its DNA target sequence must interfere with gene transcription. Each of the potential approaches listed above has its own peculiar limitations. For example, while triple helix-forming oligonucleotides have the potential for sequence selectivity and can effectively inhibit transcription *in vitro*, these molecules suffer from poor cell permeability and permeabilized cells need to be used for effective gene inhibition. Similarly, zinc finger peptides must be introduced via a gene therapy approach with an appropriate viral or non-viral expression vector since these peptides cannot directly enter cells. In contrast, the calicheamicin oligosaccharides are sufficiently hydrophobic to pass through cell membranes, but these molecules possess severely limited sequence specificity (4 bp) and bind DNA with very low affinities (100 μM or higher required for inhibition of protein-DNA interactions). Thus, new classes of cell-permeable molecules that possess higher degrees of DNA sequence specificity and affinity are needed for any human gene therapeutic approach to be feasible.

Another approach utilizes cell-permeable small molecules that target particular DNA sequences. These molecules would be useful for the regulation of gene expression. The design of small synthetic DNA-binding ligands that recognize specific sequences in the DNA double helix has been a long standing goal of chemistry. Oligodeoxynucleotides that recognize the major groove of double-helical DNA via triple-helix formation bind to a broad range of sequences with high affinity and specificity. Although oligonucleotides and their analogs have been shown to interfere with gene expression, the triple helix approach is limited to purine tracks and suffers from poor cellular uptake.

Other small molecules have also been of interest as DNA-binding ligands. Wade, *et al.* reported the design of peptides that bind in the minor groove of DNA at 5'-(A,T)G(A,T)C(A,T)-3' sequences by a dimeric side-by-side motif (*J. Am. Chem. Soc.* 114, 8783-8794 (1992)). Mrksich, *et al.* reported antiparallel side-by-side motif for sequence specific-recognition in the minor groove of DNA by the designed peptide 1-methylimidazole-2-carboxamidenetropsin (*Proc. Natl. Acad. Sci. USA* 89, 7586-7590 (1992)). Pelton, J.G. & Wemmer, D.E. reported the structural characterization of a 2-1 distamycin A-d(CGCAAATTTGGC) complex by two-dimensional NMR (*Proc. Natl. Acad. Sci. USA* 86, 5723-5727 (1989)).

Dervan and colleagues have shown that synthetic pyrrole-imidizole polyamides bind DNA with excellent specificity and very high affinities, even exceeding the affinities of many sequence-specific transcription factors (Trauger, *et al.*, *Nature* 382, 559-561 (1996)). They further describe the recognition of DNA by designed ligands at subnanomolar concentrations. DNA recognition depends on side-by-side amino acid pairing of imidizole-pyrrole or pyrrole-pyrrole pairs in the minor groove. White, S., *et al.*, (1996) reported the effects of the A•T/T•A degeneracy of pyrrole-imidazole polyamide recognition in the minor groove of DNA (*Biochemistry* 35, 6147-6152 (1996)). White, *et al.* (1997) reported pairing rules for recognition in the minor groove of DNA by pyrrole-imidazole polyamides (*Chem. & Biol.* 4, 569-578), and demonstrated the 5'-3' N-C orientation preference for polyamide binding in the minor groove. Thus, polyamide molecules thus have the potential to act as inhibitors of protein-DNA interactions in the minor groove.

The development of pairing rules for minor groove binding polyamides derived from N-methylpyrrole (Py) and N-methylimidazole (Im) amino acids provides another means to confer sequence specificity. An Im/Py pair distinguishes G•C from C•G, and both of these from A•T or T•A base pairs, while Py/Im targets a C-G basepair. A Py/Py pair specifies A•T from G•C but does not distinguish A•T from T•A. The generality of this approach to the rational design of sequence-specific DNA ligands is supported by direct NMR structural studies (Geierstanger, *et al.*, *Science* 266,646-650 (1994)) and the recent success in synthesis of an eight ring hairpin polyamide which targets a six base pair sequence with an apparent dissociation constant of 0.03 nM (Trauger, *et al.*, above). Moreover, two eight-ring pyrrole-imidizole polyamides differing in sequence by a single amino acid residue bind specifically to respective six base-pair target sites that differ in sequence by a single base pair. The replacement of a single nitrogen atom with a C-H can regulate specificity and affinity by two orders of magnitude.

Since a six base-pair sequence would be highly redundant in the human genome (occurring at random once every 4 kilobases, or 500,000 times in the human genome), polyamides have been synthesized to recognize much longer sequences. For example, a twelve-ring double hairpin polyamide has been designed to target a 12 bp site and binding is again observed with nanomolar affinity. Such a sequence would be predicted to occur at random only once every 16 million base pairs, or only 125 times in the human genome. Such molecules thus have the potential to act as specific inhibitors of gene transcription *in vivo* and as human therapeutic agents if the conditions outlined above can be met.

SUMMARY OF THE INVENTION

The present invention relates to and includes methods and compositions for the modulation, or regulation, of gene expression or overexpression by reducing the transcription of genes, particularly oncogenes. Preferably, the transcription of specific individual target oncogenes is reduced or inhibited. Such reductions result from the application of polyamides that bind or interact with the minor groove of double-stranded DNA (dsDNA) within the promoter region of a target gene. Preferably, the binding or interaction is with a predetermined target nucleic acid sequence within the promoter region to inhibit or down-regulate transcription.

Herein, "promoter" or "promoter region" refers to nucleic acid sequences needed for gene expression, and includes the region of DNA involved in binding RNA polymerase to initiate transcription of the gene associated with the promoter. It also includes sequences that bind other proteins that facilitate RNA polymerase's association with DNA in order to initiate transcription. Promoters can also include enhancer regions.

The present invention reduces gene expression and overexpression by use of sequence-specific DNA-binding small molecules that are cell-permeable and capable of inhibiting oncogene transcription. Appropriate application of such molecules may inhibit aberrant expression or activation of endogenous oncogenes to provide a fundamentally new therapeutic strategy for the treatment of various diseases, including cancer. The small molecules of the invention are polyamides that bind to or interact with nucleic acid sequences within the promoter region of target genes. Preferably, these sequences are recognized, or proximal to those that are recognized, by one or more transcription factors.

The polyamide compounds of the invention bind to dsDNA according to pairing rules for polyamide subunit recognition of nucleotide bases. More specifically, derivatives of pyrrole, imidazole, 3-hydroxypyrrole, and strategically positioned aliphatic amino acid residues form structures that recognize specific, targeted nucleotide base pairs in the minor groove of dsDNA. Selected aromatic and aliphatic amino acids may also be incorporated into polyamides, which residues remain unpaired with other amino acid residues. The polyamide molecules have a crescent-shaped form that allows them to complex with the minor groove of double-stranded DNA. It has been found by NMR studies that these compounds can bind to DNA in a 2:1 motif in which two ligands are arranged in an antiparallel way, side-by-side to each other. Pelton, J., *et al.*, *Proc. Natl. Acad. Sci. USA* **86**, 5723-5727 (1986); Mrksich, *et al.*, *Proc. Natl. Acad. Sci. USA*, **89**, 7586-7590 (1992); Wade, *et al.*,. *J. Am. Chem. Soc.* **114**, 8783 (1992).

To increase binding affinity, two polyamides can be covalently linked by a turn-unit such as γ-aminobutyric acid. Mrksich, *et al.*, *J. Am. Chem. Soc.* **116**, 7983 (1994). Such polyamides are called "hairpin polyamides", as they adopt a hairpin-like conformation in the DNA complex. The sequence of the imidazole and the pyrrole carboxamides in the polyamide determines the DNA sequence specificity of the ligand, according to the scheme of carboxamide pairs that recognize nucleotide pairs described

above. In some cases it has been useful to replace one or several pyrrole carboxamide units with β-alanine moieties in order to adjust the curvature of the polyamide to that of the DNA. It has also been found that polyamides bearing a chiral R-2,4-diaminobutyric acid instead of a γ-aminobutyric acid as the turn unit bind to DNA with a higher affinity. It has recently been shown that polyamides comprising N-methylimidazole and N-methylpyrrole carboxamides can inhibit gene expression in eukaryotic cells. Gottesfeld, *et al.*, *Nature* 387, 203-205 (1997).

It has been found that the aromatic amino acid, 3-hydroxy-N-methylpyrrole (Hp), can be incorporated into a polyamide and paired opposite Py to provide the ability to design and synthesize polyamide DNA-binding ligands that discriminate A•T nucleotide pairs from T•A nucleotide pairs. The replacement of a single hydrogen atom on the pyrrole with a hydroxy group in a Hp/Py pairing regulates affinity and specificity of a polyamide by an order of magnitude. By using Hp together with Py and Im in four pairs of aromatic amino acid residues (Im/Py, Py/Im, Hp/Py, and Py/Hp) polyamides can be designed and synthesized that selectively distinguish all four Watson-Crick base pairs in the minor groove of double stranded DNA.

The invention encompasses improved polyamides for binding to the minor groove of DNA as well as methods for the design and synthesis of specific polyamides that correspond to and bind selectively to a nucleotide sequence of a desired target DNA. The preparation and use of polyamides for binding in the minor groove of DNA are extensively described in the art. In preferred embodiments of this invention, improved polyamides are employed that utilize 3-hydroxy-N-methylpyrrole to provide Hp/Py carboxamides to bind to a T•A base pair in the minor groove of DNA or Py/Hp carboxamide binding pair in the polyamide to bind to an A•T base pair in the minor groove of DNA.

In preferred embodiments, this invention provides polyamides having three or more carboxamide binding pairs that will distinguish A•T, T•A, C•G, and G•C base pairs in the minor groove of dsDNA. The invention encompasses polyamides having γ-aminobutyric acid to form a hairpin-loop with a member of each carboxamide pairing on each side of it. Preferably the γ-aminobutyric acid is a chiral (R)-2,4-diaminobutyric acid.

The invention also encompasses polyamides containing a β-alanine substituted for a Py that would ordinarily be used in a carboxamide binding pair to match a particular nucleotide pair. The β-alanine is referred to in formulas as β. The β becomes a member of a carboxamide binding pair, and serves to optimize hydrogen bonding of neighboring amino acid moieties to nucleotide base pairs. The invention further includes the substitution as a β•β binding pair for non-Hp containing binding pair. Thus, binding pairs in addition to the Hp/Py and Py/Hp are Py/Py, Im/Py, Py/Im, Im/β, β/Im, Py/β, β/Py, and β/β.

In general, the invention provides polyamides suitable for inhibiting the transcription of an endogenous oncogene, wherein the polyamides comprise at least three complementary pairs of aromatic carboxamide residues, the complementary pairs of aromatic carboxamide residues being selected to correspond to the nucleotide sequence of an identified dsDNA target, including at least two aliphatic amino acid residues chosen from the group consisting of glycine, β-alanine, γ-aminobutyric acid, R 2,4-diaminobutyric acid, and 5-aminovaleric acid, and at least one terminal alkylamino residue. The complementary pairs of aromatic carboxamide residues selected to correspond to the nucleotide sequence of an identified dsDNA target are chosen from the group consisting of Im/Py to correspond to the nucleotide pair G/C, Py/Im to correspond to the nucleotide pair C/G, Py/Py to correspond to the nucleotide pair A/T, Py/Py to correspond to the nucleotide pair T/A, Hp/Py to correspond to the nucleotide pair T/A, and Py/Hp to correspond to the nucleotide pair A/T, where Im is N-methyl imidazole, Py is N-methyl pyrrole and Hp is 3-hydroxy N-methyl pyrrole. As those in the art will appreciate, the polyamides of the invention can also be used to prevent, inhibit, or reduce transcription from a viral oncogene.

Preferably the polyamides bind to the minor groove of double-stranded DNA in a promoter region that controls the transcription and expression of an oncogene. In preferred embodiments, the polyamides bind to the nucleotide sequence of an identified dsDNA target chosen from the group consisting of 5'-TGCTTGA-3', 5'-AGAATGA-3', 5'-TGAGGAA-3', 5'-TGCTTGA-3', 5'-TGAGGAA-3', 5'-AGGAAGT-3', 5'-ATGAAGT-3', 5'-AGTATAA-3', 5'-AGTATAA-3', 5'-AGGAAGT-3', 5'-AGTATAA-3', 5'-AGTATAA-3', 5'-AACGGCT-3', 5'-TGCAGGCA-3', 5'-AACGGCT-3', 5'-TGCAGGCA-3', and 5'-AGGCAA-3'. Preferably the transcription of the gene is

inhibited by modulating the binding of a protein transcription factor to dsDNA. In preferred embodiments, the transcription factors are ESX, ETS, AP-2, OB2-1, and TBP.

Preferred polyamides comprise at least one aliphatic amino acid residue is β-alanine. In preferred embodiments, the terminal alkylamino residue is a N,N-dimethylaminopropyl residue. Suitable polyamides can contain at least two β-alanine residues aligned to form complementary paired residues corresponding to a nucleotide pair chosen from the group A/T and T/A. Alternatively, corresponding pairs can be formed between aliphatic amino acids and aromatic carboxamides, such as Im/β, β/Im, Py/β and β/Py. In preferred polyamides, a hairpin molecule is formed by a an aliphatic amino acid residue such as γ-aminobutyric acid or more preferably R 2,4-diaminobutyric acid.

Suitable polyamides have a binding affinity at the dsDNA target sequence of at least 10^9 M^{-1} and a selectivity of at least about two, selectivity being defined as the ratio of the binding affinity for the identified dsDNA target sequence to the binding affinity for a single base-pair mismatch dsDNA sequence. In preferred embodiments, selectivity against at least 90% of single base mismatch sequences is greater than about 10.

The present invention provides polyamides suitable for inhibiting the transcription of an oncogene comprising at least three complementary pairs of aromatic carboxamide residues, the complementary pairs of aromatic carboxamide residues being selected to correspond to the nucleotide sequence of an identified dsDNA target, at least two aliphatic amino acid residues chosen from the group consisting of glycine, β-alanine, γ-aminobutyric acid, R 2,4-diaminobutyric acid, and 5-aminovaleric acid, and at least one terminal alkylamino residue, the polyamide having a binding affinity at the target dsDNA sequence of at least 10^9 M^{-1} and a selectivity of at least about two, selectivity being defined as the ratio of the binding affinity for the identified target dsDNA sequence to the binding affinity for a single base-pair mismatch dsDNA sequence.

In a related aspect, the invention concerns compositions comprising a pharmaceutically acceptable excipient and a transcription-inhibiting amount of at least one polyamide according to the invention. Preferably, the polyamides bind to the minor groove of double-stranded DNA in a promoter region that controls the transcription and

expression of a gene, particularly an oncogene. Preferably the transcription of a selected oncogene is inhibited by modulating the binding of a protein transcription factor to dsDNA, preferably in the gene's promoter region. In preferred embodiments, the transcription factors are ESX, ETS, AP-2, OB2-1,and TBP.

Previous studies directed toward inhibition of the transcriptional activity of the HIV-1 promoter have demonstrated that polyamides can block binding of TBP as well as an *Ets* family transcription factor (see PCT published application PCT/US98/02444, now WO 98/35702, the teachings of which are incorporated by reference as if fully set forth). In principle, both classes of transcription factors can be inhibited by polyamides that contact or bind the minor groove of dsDNA. DNA complexation of proteins contacting the minor groove may be inhibited by direct steric hindrance, repulsion, or exclusion or, alternatively, by allosteric effects. For example, the binding of major groove binding proteins may be suppressed by a polyamide-induced change of the DNA conformation. Of course, inhibition can also be achieved other ways, for example, by conjugating a DNA cleavage agent to a polyamide targeted to a desired site, or by chemically modifying DNA using a chemically reactive moiety attached to a polyamide.

Thus, in another aspect of the invention, the expression or overexpression of oncogenes is reduced or inhibited by using a polyamide targeted to a specific nucleotide base sequence in dsDNA, particularly a target sequence in the promoter region of the particular oncogene. Preferably, the oncogenes are viral or endogenous cellular oncogenes involved in cancer. One oncogene target of the invention is the HER-2/neu gene, which may be down-regulated or inhibited by the use of polyamides that bind to target sequences within the HER-2/neu promoter region. Preferably, the sequences targeted by a polyamide are, or are proximal to, transcription factor binding sites within the oncogene promoter. Interactions or binding between the polyamide and the target sequence can inhibit the transcription of the targeted oncogene. The degree of inhibition of oncogene expression can be extensive, and includes the inhibition of overexpression, such that a level of expression correlated with a non-disease state is restored. Of course, even greater inhibition may be achieved, depending on the particular application. The invention further encompasses application of polyamides for the treatment of various tumors or cancers (including breast cancer) associated with oncogene expression.

Suitable polyamides most preferably have a binding affinity at the dsDNA target sequence of at least 10^9 M^{-1} and a selectivity of at least about two. Selectivity is defined as the ratio of the binding affinity for the identified dsDNA target sequence to the binding affinity for a single base-pair mismatch dsDNA sequence. In preferred embodiments, selectivity against at least 90% of single base mismatch sequences is greater than about 10.

In a related aspect of the present invention, compositions are provided that comprise a pharmaceutically acceptable excipient and a transcription-inhibiting amount of at least one polyamide of the invention. Each polyamide contains at least three complementary pairs of aromatic carboxamide residues, which pairs are selected to correspond to an identified nucleotide sequence of a dsDNA target. Preferably, the polyamides additionally comprise at least two aliphatic amino acid residues chosen from the group consisting of glycine, β-alanine, γ-aminobutyric acid, R-2,4-diaminobutyric acid, and 5-aminovaleric acid, and at least one terminal alkylamino residue, the polyamide having a binding affinity at the target dsDNA sequence of at least 10^9 M^{-1} and a selectivity of at least about two, selectivity being defined as the ratio of the binding affinity for the identified target dsDNA sequence to the binding affinity for a single base-pair mismatch dsDNA sequence.

The invention further provides methods suitable for treating a subject having a condition associated with abnormal expression of a cellular oncogene. The subject is preferably a human patient and, more particularly, one afflicted with breast cancer or other diseases or conditions associated with aberrant Her-2/neu oncogene expression.

Brief Description of the Drawings

Figure 1 depicts the HER2/neu promoter, showing the nucleotide sequence in A, including binding sites of ESX, AP-2, OB2-1, and TBP ("TATA") transcription factors, and in B, a schematic diagram, not to scale, showing the ESX, TBP, and OB2-1 binding sites.

Figure 2 in A shows the nucleotide sequence of the ESX binding site, indicating four overlapping polyamide target sites, and in B, the schematic structure of eight

polyamides (**1-8**) that have been designed to bind to the nucleotide sequences of these target sites 1-4, in which N-methylimidazole carboxamides are represented by filled circles, N-methylpyrrole carboxamides by empty circles, and β-alanine amino acids by unfilled diamonds. 2, 4-diaminobutyric acid is represented by a curved line bearing an amino group and the N,N-dimethylaminopropyl substituent (and the C-terminus) by a positively charged half-circle.

Figure 3 illustrates a typical solid-phase synthesis scheme, using polyamide **1** as an example.

Figure 4 shows the structural formulas of eight polyamides that have been designed to bind to the ESX binding site, as well as MALDI-MS data that characterize each compound.

Figure 5 is a graphical representation of the results of a DNase I footprint titration of polyamide **1** on the 188 base-pair 5'-end-labeled DNA fragment, showing in A an autoradiogram: lane 1, A reaction; lane 2 to 12, 20 pM, 40 pM, 80 pM, 100 pM, 200 pM, 400 pM, 1 nM, 2 nM, 4 nM 10 nM, 20 nM polyamide **1**, all reactions containing 15 kcpm DNA fragment, 10 mM Tris HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, the match- and the single base-pair mismatch site shown to the right side of the autoradiogram, and in B, the schematic structure of polyamide **1**.

Figure 6 summarizes the results of DNase I footprint titrations, showing in A, a portion of the nucleotide sequence of the HER2/neu promoter containing the ESX binding site with polyamide binding sites 1-4 indicated, and in B, the four polyamide target sites with corresponding schematic representations of polyamides **1-8**.

Figure 7 in A shows the nucleotide sequence of a AP-2 binding site, indicating three overlapping polyamide target sites, and in B, the schematic structure of five polyamides (**9-13**) that were designed to bind to the nucleotide sequences of these target sites 1-3.

Figure 8 shows the structural formulas of five polyamide compounds designed to bind to the nucleotide sequence of the AP-2 binding site, as well as MALDI-MS data that characterize each compound.

Figure 9 is a graphical representation of the results of a DNase I footprint titration of polyamide 9 on the 188 base-pair 5'-end-labeled DNA fragment, showing in A an autoradiogram: lane 1, A reaction; lane 2 to 12, 20 pM, 40 pM, 80 pM, 100 pM, 200 pM, 400 pM, 1 nM, 2 nM, 4 nM 10 nM, 20 nM polyamide **9**, all reactions containing 15 kcpm DNA fragment, 10 mM Tris HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, and in B, the schematic structure of polyamide **9**.

Figure 10 is a graphical representation of the results of a DNase I footprint titration of polyamide **10** on the 188 base-pair 5'-end-labeled DNA fragment, showing in A an autoradiogram: lane 1, A reaction; lane 2 to 12, 20 pM, 40 pM, 80 pM, 100 pM, 200 pM, 400 pM, 1 nM, 2 nM, 4 nM 10 nM, 20 nM polyamide **10**, all reactions containing 15 kcpm DNA fragment, 10 mM Tris HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, the match site shown to the right side of the autoradiogram, and in B, the schematic structure of polyamide **10**.

Figure 11 is a graphical representation of the results of a DNase I footprint titration of polyamide **13** on the 188 base-pair 5'-end-labeled DNA fragment, showing in A an autoradiogram: lane 1, A reaction; lane 2 to 12, 20 pM, 40 pM, 80 pM, 100 pM, 200 pM, 400 pM, 1 nM, 2 nM, 4 nM 10 nM, 20 nM polyamide **13**, all reactions containing 15 kcpm DNA fragment, 10 mM Tris HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, the match site shown to the right side of the autoradiogram, and in B, the schematic structure of polyamide **13**.

Figure 12 summarizes the results of DNase I footprint titrations, showing in A a portion of the nucleotide sequence of the HER2/neu promoter containing the AP-2 binding site with polyamide binding sites 1-3 indicated, and in B, the three polyamide target sites with corresponding schematic representations of polyamides **9-13**.

Figure 13 is a graphical representation of the results of a gel shift experiment showing the binding of an oligonucleotide containing the ESX binding site of the

HER2/neu promoter and the ESX protein in the presence of various concentrations of polyamide 2 ("PA-2E") or distamycin.

Figure 14 is a graphical representation of a quantitative comparison of the inhibition of the ESX/DNA complex by polyamide 1 ("PA-1E") and polyamide 2 ("PA-2E") presented before exposure to ESX ("Before") or after the formation of the ESX/DNA complex ("After").

Figure 15 is a graphical representation of a quantitative comparison of the ability of polyamides 1 (A) and 2 (B) to inhibit *in vitro* transcription driven by the HER2/neu promoter when the respective polyamides were presented before exposure to ESX ("Before") or after the formation of the ESX/DNA complex ("After").

Figure 16 is a graphical representation of a quantitative comparison of the inhibition of the ESX/DNA complex by polyamide 2 ("PA-2E") or distamycin ("Dist").

Figure 17 is a graphical representation of a quantitative comparison of the inhibition of the formation of the ESX/DNA complex or the AP-2/DNA complex by polyamide 1 ("PA-1E"), polyamide 2 ("PA-2E") or distamycin.

Figure 18 is a graphical representation of a quantitative comparison of the inhibition of the formation of the ESX/DNA complex by polyamide 1 ("PA-1E"), polyamide 2 ("PA-2E") or polyamide 3 ("PA-3E").

Figure 19 is a graphical representation of a quantitative comparison of the ability of polyamide 1 ("PA-1E"), polyamide 2 ("PA-2E"), polyamide 3 ("PA-3E") or distamycin (Dist")to inhibit *in vitro* transcription driven by the HER2/neu promoter when the respective polyamides were presented before exposure to ESX.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to methods and compositions for modulating or regulating oncogene expression or overexpression by reducing or inhibiting oncogene transcription, especially of oncogenes involved in cancer, particularly human cancer. The

reduction in gene transcription results from binding or other interactions between polyamides and the minor groove of dsDNA within the promoter regions of target oncogenes. Preferably, the polyamides bind or interact with specific target nucleic acid sequences within the promoter regions to inhibit or down-regulate oncogene transcription. Preferably, the sequences are recognized, or proximal to those that are recognized, by one or more transcription factors that bind to or otherwise functionally interact with the promoter region of the targeted oncogene.

As used herein, "oncogene" refers to a gene that causes transformation (*i.e.*, conversion to a tumorigenic, or oncogenic, state) upon expression in a cell. Oncogenes can be viral in origin, or they may arise from a mutation (including point mutations (*e.g.*, substitutions, deletions, and insertions), chromosomal rearrangement (*e.g.*, an insertion, translocation, and copy number amplification), or aberrant expression of an endogenous, cellular proto-oncogene. At present, about 100 oncogenes have been identified. Oncogenes are classified based on activity, and include those that are transmembrane proteins (*e.g.*, cell surface receptors) to transcription factors and other nuclear proteins (*e.g.*, tumor suppressors, for example, p53). The generation of an oncogene from a proto-oncogene represents a change of function, more typically, a gain or loss of function, wherein the gene is inappropriately activated, as may be caused by a mutation, constitutive expression, over-expression, or inappropriate regulation of expression.

Many oncogenic animal viruses are known, and include both DNA and RNA viruses. Examples include polyomaviruses, adenoviruses, hepatitis viruses, and retroviruses. Representative examples of retroviral oncogenes, and the disease(s) with which they are associated (*see* brackets), include *src* (sarcoma), H-*ras* (sarcoma, erythroleukemia), K-*ras* (sarcoma, erythroleukemia), *mos* (sarcoma), *fos* (chondrosarcoma), *sis* (sarcoma), *fms* fibrosarcoma), *fes* (fibrosarcoma), *jun* (fibrosarcoma), *fps* (sarcoma), *myc* (carcinoma, sarcoma, myelocytoma), *abl* (B cell leukemia), *rel* lymphatic leukemia), *erb*A (erythroleukemia, fibrosarcoma), *erb*B (erythroleukemia, fibrosarcoma), and *myb* (myeloblastic leukemia). Other oncogenes include *rel* and N-*ras*. Representative examples of cellular oncogenes include *c-myc*, *c-abl*, *c-myb*, *c-erbB*, *c-K-ras*, and *mdm2*, all of which are amplified (in terms of gene copy number) in the various tumors with which they are associated. Alternatively, the expression of an oncogene may be elevated in certain tumors, although the gene's coding

sequence is unchanged, as occurs with respect to *c-myc* in some tumors. Expression of *c-myc* is known to be increased by several mechanisms, for example, by retroviral insertion in the gene's vicinity. Increased *c-myc* expression is believed to result by constitutive transcription initiated from the viral long terminal repeat (LTR) by virtue of its efficient promoter sequence or enhancer. Other cellular oncogenes activated by the integration of a retroviral genome include, without limitation, *c-erbB*, *c-myb*, *c-mos*, *c-raf*, *c-wnt1*, *c-int2*, and *c-H-ras*. *c-myc* expression is also known to be increased in some tumors, particularly those of the immune system, as a result of a translocation, as well as by gene amplification.

The gene products encoded by oncogenes or proto-oncogenes have a variety of cellular functions. Some of these gene products are growth factors (*e.g.*, *c-sis*, *KS/HST*, *wnt1*, and *int2*), growth factor receptors (*e.g.*, the viral, oncogenic counterparts of *c-erbB*, *c-erbB*-2 (also known as HER2/neu), *erb2,3*, *c-fms*, *c-kit*, and *mas*), signal transduction proteins (*e.g.*, *c-ras*), intracellular tyrosine kinases (*e.g.*, *c-src*, *c-abl*, *c-fps*, *v-src*, *v-yes*, *v-fgr*, *v-fps/fes*, *v-abl*, and *v-ros*), intracellular serine/threonine kinases (*e.g.*, *c-raf* and *c-mos*), signaling proteins (*e.g.*, *crk* and *vav*), or transcription factors (*e.g.*, *c-myc*, *c-myb*, *c-fos*, *c-jun*, *c-rel*, and *c-erbA*).

As will be appreciated by those in the art, a polyamide according to the invention, and compositions and methods of using the same, can be applied to inhibit or otherwise down-regulate the expression of any oncogene, whether now known or later discovered. In short, the recognition and/or binding sequences of DNA binding proteins that influence regulation of a particular oncogene are identified, polyamides targeted to these sequences are designed and synthesized, after which they can be incorporated into the disclosed compositions, if desired, and be used to inhibit transcription of the desired oncogene, be it *in vivo* or *in vitro*.

The polyamides are preferably cell-permeable and capable of inhibiting gene transcription *in vivo*, *in vitro*, or in cell free systems. Appropriate application of such polyamide molecules may be used to inhibit expression or overexpression of viral or endogenous oncogenes as a treatment of various diseases, including cancer.

In preferred embodiments, the polyamides bind to the minor groove of double stranded DNA in a promoter region that controls the transcription and expression of a target oncogene. Preferred target oncogenes are endogenous oncogenes involved in cancer formation or progression. Also preferred are oncogenes encoded by the genomes of viruses that infect animals, particularly mammals, and especially humans and/or bovine, canine, equine, feline, ovine, and/or porcine animals. Preferably, the transcription of the oncogene is inhibited by modulating the binding of a protein, such a transcription factor, to the same promoter region with which the polyamide binds or interacts. In especially preferred embodiments, the transcription factors are one or more of the following: ESX; ETS; AP-2, and TBP.

Inhibition of transcriptional activity at the HIV-1 promoter demonstrates that polyamides can block binding of TBP as well as an *Ets* family transcription factor. *See* WO 98/35702, which also includes a discussion of polyamide synthesis. The present invention includes the use of polyamides that inhibit or modulate the activity of a transcription factor with its particular recognition and/or binding sequence. The methods of the invention affect transcription factor activity by use of one or more polyamides that contact or bind the minor groove of dsDNA. Such contact or binding may inhibit formation of DNA-transcription factor complexes in the minor groove by direct steric repulsion, allosteric effects, or other mechanisms (*e.g.*, cleavage or chemical modification of the dsDNA). In addition, the binding of major groove DNA binding proteins, such as TBP, may be inhibited by a polyamide-induced change in DNA conformation through association with the neighboring minor groove.

In a preferred aspect of the invention, the expression or overexpression of oncogenes, especially viral or endogenous cellular oncogenes, is targeted. Preferably, the oncogenes are those implicated in cancer, and their expression or overexpression is inhibited by polyamides that contact or bind the minor groove in the region of the oncogene promoter. Preferably, the contacted or bound portions of the promoter region are, or are proximal to, transcription factor binding sites. The degree of inhibition is preferably large and more preferably enough to inhibit even overexpression of the oncogene, in when the copy number of the gene increases.

One oncogene target of the invention is the HER-2/neu gene, which may be down-regulated or inhibited by the use of polyamides that bind to target sequences within the HER-2/neu promoter region. Preferably, these sequences are, or are proximal to, transcription factor binding sites within the HER2/neu promoter. These transcription factors include TBP, ESX, and AP-2. Interactions or binding between the polyamide and the target sequence result in inhibition of the HER2/neu gene transcription.

The present invention includes compositions comprising a pharmaceutically acceptable excipient and a transcription-inhibiting amount of at least one polyamide for the inhibition of gene expression or overexpression. These compositions may also be used for the treatment of various tumors or cancers, including breast cancer. The invention further provides methods of administering such compositions to result in inhibition of gene expression or overexpression. The methods and compositions are preferably suited for treating a subject having a condition associated with abnormal expression of a cellular oncogene. The subject is preferably a human patient particularly one afflicted with cancer, especially breast cancer.

Design of the Polyamides.

The nucleotide sequence of the region of the HER2/neu promoter encompassing the ESX binding site is shown in Figure 2A. This region was divided into four overlapping target sites, each consisting of 7 base-pairs. Two polyamides consisting of N-methylimidazole carboxamide residues, N-methylpyrrole carboxamide residues, and none, one or two β-alanine aliphatic amino acid residues were designed to correspond to the DNA sequence of each of these target sites (Fig. 2B). The sequence of the N-methylimidazole carboxamide residues and N-methylpyrrole carboxamide residues was chosen according to the pairing rules described above. Polyamide **1** had the structure ImPy-β-PyIm-2,4DA-PyPy-β-ImPy-β-Dp. Polyamide **2** had the structure ImPyPyPyPy-2,4DA-PyPyPyPyPy-β-Dp. Polyamide **3** had the structure ImPy-β-PyPy-2,4DA-PyPy-β-PyPy-β-Dp. Polyamide **4** had the structure ImPyPyPyIm-2,4DA-PyPyPyImPy-β-Dp. Polyamide **5** had the structure ImIm-β-PyIm-2,4DA-PyPy-β-PyPy-β-Dp. Polyamide **6** had the structure ImIm-β-PyIm-2,4DA-PyPyPyPyPy-β-Dp. Polyamide **7** had the structure Im-β-ImImPy-2,4DA-PyPyPy-β-Py-β-Dp. Polyamide **8** had the structure Im-β-ImImPy-2,4DA-PyPyPyPyPy-β-Dp.

Preparation of the Polyamides.

The building blocks that are required for the solid-phase synthesis of the polyamides were prepared on large scales (50 to 200 g) according to established procedures. All polyamides were prepared by solid-phase synthesis on a Boc-β-alanine-PAM resin which is commercially available in appropriate substitution levels (0.615 mmol/g). The synthesis of the polyamide **1** is depicted in Figure 3 as a representative example. All polyamides were characterized by MALDI-MS; the experimentally obtained data were in good agreement with the expected values. Figure 4 shows the structural formulas of eight polyamides that have been designed to bind to the ESX binding site, as well as MALDI-MS data that characterize each compound.

Pharmaceutical and therapeutic compositions

The polyamides of the invention, as well as the pharmaceutically acceptable salts thereof, may be formulated into pharmaceutical or therapeutic compositions, formulations, or preparations. Pharmaceutically acceptable salts of the polyamide compounds of the invention are formed where appropriate with strong or moderately strong, non-toxic, organic, or inorganic acids or bases by methods known in the art. Exemplary of the salts that are included in this invention are maleate, fumarate, lactate, oxalate, methanesulfonate, ethanesulfonate, benzenesulfonate, tartrate, citrate, hydrochloride, hydrobromide, sulfate, phosphate, and nitrate salts.

As stated above, the polyamide compounds of the invention possess the ability to inhibit oncogene expression or overexpression, properties that are exploited in the treatment of any of a number of diseases or conditions, most notably cancer. A composition of this invention may be active *per se*, or may act as a "pro-drug" that is converted *in vivo* to an active form.

The compounds of the invention, as well as the pharmaceutically acceptable salts thereof, may be incorporated into convenient dosage forms, such as capsules, impregnated wafers, tablets, or injectable preparations. Solid or liquid pharmaceutically acceptable carriers may be employed. Pharmaceutical compositions designed for timed release may also be formulated.

Preferably, the compounds of the invention are administered systemically, *e.g.*, by injection. When used, injection may be by any known route, preferably intravenous, subcutaneous, intramuscular, intracranial, or intraperitoneal. Injectables can be prepared in conventional forms, either as solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions.

Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate and stearic acid. Liquid carriers include syrup, peanut oil, olive oil, saline, water, dextrose, glycerol and the like. Similarly, the carrier or diluent may include any prolonged release material, such as glyceryl monostearate or glyceryl distearate, alone or with a wax. When a liquid carrier is used, the preparation may be in the form of a syrup, elixir, emulsion, soft gelatin capsule, liquid containing capsule, sterile injectable liquid (*e.g.*, a solution), such as an ampoule, or an aqueous or nonaqueous liquid suspension. A summary of such pharmaceutical compositions may be found, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton Pennsylvania (Gennaro 18th ed. 1990).

The pharmaceutical preparations are made following conventional techniques of pharmaceutical chemistry involving such steps as mixing, granulating and compressing, when necessary for tablet forms, or mixing, filling and dissolving the ingredients, as appropriate, to give the desired products for oral or parenteral, including topical, transdermal, intravaginal, intranasal, intrabronchial, intracranial, intraocular, intraaural and rectal administration. The pharmaceutical compositions may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and so forth.

Although the preferred routes of administration are systemic, the pharmaceutical composition may be administered topically or transdermally, *e.g.*, as an ointment, cream or gel, orally, rectally, *e.g.*, as a suppository, parenterally, by injection or continuously by infusion, intravaginally, intranasally, intrabronchially, intracranially intra-aurally, or intraocularly.

For topical application, the composition may be incorporated into topically applied vehicles such as a salve or ointment. The carrier for the active ingredient may be

either in sprayable or nonsprayable form. Non-sprayable forms can be semi-solid or solid forms comprising a carrier indigenous to topical application and having a dynamic viscosity preferably greater than that of water. Suitable formulations include, but are not limited to, solution, suspensions, emulsions, creams, ointments, powders, liniments, salves, and the like. If desired, these may be sterilized or mixed with auxiliary agents, *e.g.*, preservatives, stabilizers, wetting agents, buffers, or salts for influencing osmotic pressure and the like. Preferred vehicles for non-sprayable topical preparations include ointment bases, *e.g.*, polyethylene glycol-1000 (PEG-1000), conventional creams such as HEB cream, gels, as well as petroleum jelly and the like.

Also suitable for topical application are sprayable aerosol preparations wherein the compound, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, perfumes, and/or antioxidants in addition to the compounds of the invention.

For preferred topical applications, especially for humans, it is preferred to administer an effective amount of the compound to a target area, *e.g.*, skin surface, mucous membrane, eyes, *etc.* This amount will generally range from about 0.001 mg to about 1 g per application, depending upon the area to be treated, the severity of the symptoms or disease, and the nature of the topical vehicle employed.

The compositions of the invention can also be administered in combination with one or more additional compounds that are used to treat the disease or condition. For treating cancer, the polyamides and derivatives are given in combination with anti-tumor agents, such as mitotic inhibitors, *e.g.*, vinblastine; alkylating agents, *e.g.*, cyclophosphamide; folate inhibitors, *e.g.*, methotrexate, pritrexim or trimetrexate, antimetabolites, *e.g.*, 5-fluorouracil and cytosine arabinoside, intercalating antibiotics, *e.g.*, adriamycin and bleomycin, enzymes or enzyme inhibitors, *e.g.*, asparaginase, topoisomerase inhibitors, *e.g.*, etoposide, or biological response modifiers, *e.g.*, interferon. In fact, pharmaceutical compositions comprising any known cancer therapeutic in combination with the polyamine analogues and derivatives disclosed herein are within the scope of this invention.

Typical single dosages of the compounds of this invention are between about 1 ng and about 10 g/kg body weight. The dose is preferably between about 0.01mg and about 1g/kg body wt. and, most preferably, between about 0.1mg and about 100mg/kg body wt. For topical administration, dosages in the range of about 0.01-20% concentration of the compound, preferably 1-5%, are suggested. A total daily dosage in the range of about 1-500 mg is preferred for oral administration. The foregoing ranges are, however, suggestive, as the number of variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are expected and may be routinely made by those skilled in the art.

Effective amounts or doses of the compound for treating a disease or condition can be determined using recognized *in vitro* systems or *in vivo* animal models for the particular disease or condition. In the case of cancer, many art-recognized models are known and are representative of a broad spectrum of human tumors. The compositions may be tested for inhibition of tumor cell growth in culture using standard assays with any of a multitude of tumor cell lines of human or non-human animal origin. Many of these approaches, including animal models, are described in detail in Geran, R.I. *et al.*, "Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems (Third Edition)", *Canc. Chemother. Reports*, Part 3, 3:1-112.

Administration methods

As noted above, the treatment methods of the invention are directed to the administration of polyamide-containing compositions. The polyamide-containing preparations of the invention may be administered systemically or locally and may be used alone or as components of mixtures. The route of administration may be topical, intravenous, oral, or by use of an implant. For example, polyamides may be administered by means including, but not limited to, topical preparations, intravenous injection or infusion, oral intake, or local administration in the form of intradermal injection or an implant. Additional routes of administration are subcutaneous, intramuscular, or intraperitoneal injections of the polyamides in conventional or convenient forms. Liposomal or lipophilic formulations may also be used when desired. For topical administration, the polyamides may be in standard topical formulations and compositions including lotions, suspensions or pastes. Oral administration of suitable formulations

may also be appropriate in those instances where the polyamides may be readily administered to the target cells or tissues via this route.

The dose of polyamides may be optimized by the skilled artisan depending on factors such as, but not limited to, the polyamides chosen, the physical delivery system in which it is carried, the individual subject, and the judgment of the skilled practitioner.

Having now generally described the invention, the same will be more readily understood through reference to the following experimental section which is provided to illustrate, but not limit the scope of, the present invention, unless specified.

DNA binding studies.

A plasmid containing the HER2/neu promoter, from which was prepared a 5'-^{32}P end-labeled 188 base-pair DNA fragment containing the ESX binding site by PCR (polymerase chain reaction).

Quantitative DNase I footprint titrations (10mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, pH 7.0 and 22–24 C) were performed to determine the equilibrium association constants (K_a) of each polyamide (**1-8**) to its respective match sequence. The autoradiogram of the titration of the polyamide **1** is shown as a representative example in Figure 5A.

Results of the quantitative DNase I footprint titrations.

The equilibrium binding constants determined from DNase I footprinting experiments for the polyamides **1** to **8** are summarized in Figure 6.

At site 1, the "footprints" of the polyamide **1** at the match and the mismatch site can be seen in the autoradiogram shown in Figure 5A. Compound **1** bound with subnanomolar affinity (K_a = 1.8 x 10^{10} M^{-1}) to its match site (5'-TGCTTGA-3'). However, polyamide **1** was not a very selective ligand, since it bounds with only about three-fold less affinity to the formal single base-pair mismatch site (5'-AGAATGA-3'). It should be noted that binding of **1** to this mismatch site may also lead to a decreased

binding of the ETS protein. Polyamide 4, which does not contain β-alanine units, showed only weak binding to both the match and the mismatch site, in both cases at [5] of about 5 nM.

At site 2, the polyamide 5 showed no significant binding at concentrations of less than 10 nM. Similarly, polyamide 6 did not bind to its designated match site (5'-TGAGGAA-3') at concentrations smaller than 10 nM. However, 6 showed several footprints at other locations of the DNA fragment at concentrations higher than 1 nM.

At site 3, polyamide 7 bound to a single base-pair mismatch site (ATGAAGT) with an association constant K_a of *ca.* 10^9 M^{-1}. No binding of polyamide 7 to the match site (5'-AGGAAGT-3') was observed (at [7] < 10 nM). Polyamide 8 bound weakly to its match site (K_a ~5 nM). However, it did not bind very selectively to the DNA fragment; among other sites it bound to the formal single base-pair mismatch site 5'-ATGAAGT-3' with an association constant K_a of *ca.* 10^9 M^{-1}.

At site 4, both polyamides 2 and 3 bound selectively to their match site (5'-AGTATAA-3') with similarly good association constants (K_a (2) = 9.5 x 10^9 M^{-1}; K_a (3) = 1.5 x 10^{10} M^{-1}). The poor binding of polyamides 5 to 8 that were designed to bind to the sites 2 and 3 of the promoter, respectively, may be due to a deviation of the DNA conformation from the B-form in that region.

The AP-2 binding site

While the transcription factor AP-2 is barely detectable in cells with low HER2/neu expression phenotype, its concentration is clearly higher in HER2/neu overexpressing cells. These findings demonstrate the important role for this transcription factor in human breast cancer.

Design of the Polyamides.

The DNA sequence of the AP-2 binding site and adjacent regions is shown in Figure 7A. As in the studies of the ESX binding site, this region was divided into three target sites, each consisting of 6 to 8 base-pairs. For each of these sites, two polyamides were prepared consisting of N-methylimidazole carboxamide residues, N-methylpyrrole

carboxamide residues, and none, one or two β-alanine amino acid residues (Figure 7B). The sequence of the N-methylimidazole carboxamide residues, N-methylpyrrole carboxamide residues, and β-alanine amino acid residues was chosen according to the pairing rules described above. Polyamide **9** had the structure ImPy-β-ImPy-2,4DA-PyPyImImPy-β-Dp. Polyamide **10** had the structure ImPy-β-ImPy-2,4DA-Py-β-ImImPy-β-Dp. Polyamide **11** had the structure ImPyPy-β-ImPy-2,4DA-ImPy-β-ImImPy-β-Dp. Polyamide **12** had the structure ImPy-β-ImImPy-2,4DA-ImPyPy-β-ImPy-β-Dp. Polyamide **13** had the structure ImImPyPy-2,4DA-PyImPyPy-β-Dp.

Synthesis of the polyamides.

The polyamides were prepared by solid-phase synthesis. Their purity was confirmed by analytical HPLC and their structures confirmed by MALDI-MS. The structures are shown in Figure 8.

The DNA binding studies.

A 5'-^{32}P end-labeled 189 base-pair DNA fragment containing the AP-2 binding site was obtained by PCR from the HER2/neu plasmid described above. Quantitative DNase I footprint titrations (10mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, pH 7.0 and 22–24 degrees C) were performed to determine the equilibrium association constants (K_a) of each polyamide (**9-13**) to its respective match sequence. In the Figures 9 to 11, the autoradiograms of the titration of the polyamide **9**, **10**, and **13** are shown as representative examples. The results are summarized in Figure 12.

While the polyamide **9** did not bind at all to site 1, polyamide **10**, which contains 2 β-alanine units, bound with a very high affinity ($K_a = 8.7 \times 10^{10}\ M^{-1}$) and selectivity to this site. Both polyamides **11** and **12** that were designed to bind to site 2 did not show a very high selectivity. The eight ring polyamide **13** bound in nanomolar concentrations and selectively to its respective match site.

Polyamides **1**, **2** and **3** were studied in greater detail and compared to the known compound distamycin.

Cell Culture

SKBR-3 cells were purchased from ATCC (Rockville, MD). Cells were grown in McCoy's 5a medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum and cultured at 37 °C with 5% CO_2.

Nuclear Extract preparation

SKBR-3 cells that were grown to subconfluence were rinsed twice with Phosphate-buffered saline, scraped, and collected by centrifugation at 1,200 rpm for 5 min., 4 °C (Sorvall RT6000, Newtown, CT). The following steps were performed at 4 °C. Cell pellets were suspended in five times the packed cell volume in buffer A (containing 10 mM Hepes-KOH [ph 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA. 0.75 mM spermidine, 0.15 mM spermine, and 1 mM dithiothreitol), followed by centrifugation at 1,200 rpm from 5 min. The pellet was then resuspended in five times the pellet volume in buffer A, kept on ice for 8 min., and homogenized with 10 strokes using a Dounce homogenizer (tight pestle). The homogenate (~ 95% lysed cells) was centrifuged at 15,000 rmp for ~ 1 min., (JA-17 rotor, JA-21 centrifuge; Beckman, Palo Alto, CA). The pellet was resuspended in buffer B and 20 mM Hepes-KOH (ph 7.9), 20% glycerol, 0.2 mM EDTA, 2.0 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 2 mM dithiothreitol, and 1 mM phenylmethylsufonyl fluoride followed by drop addition of an equal volume of buffer B that included 0.75 M NaCl. After rocking for 20 min. supernatant was collected by centrifugation at 47,500 rmp for 45 min (SW-55 rotor, Beckman), and dialyzed against > 100-fold buffer C (20 mM Hepes-KOH [pH 7.9], 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mM phenylmethysulfonyl fluoride) for 3h. Precipitated debris was removed by centrifugation at 15,000 rpm (JA-21 centrifuge, JA-17 rotor, Beckman) and the protein content of the nuclear extract was quantitated using the Bio-Rad protein assay.

Proteins and Antibodies

Bacterial-expressed ESX protein was prepared as described [Chang *et al.*, 1997]. Briefly, full-length ESX cDNA was cloned into a pRSET his-tag expression plasmid (NheI-HindIII; Invitrogen). Expression of ESX protein was processed by transformation of bacterial cells (BL21[DE3] pLysS competent bacterial cells: Stratagene, La Jolla,

CA), and induced by addition of IPTG. His-tagged ESX protein was purified using nickel-chelate affinity chromatography as recommended by the manufacturer (Quiagen, Inc., Chatsworth, CA). AP-2 protein was purchased from Promega Co. (Madison, WI). Antibodies against AP-2 were purchased from Santa Cruz Co. (La Jolla, CA), and antibodies against ESX were prepared as described previously.

Oligonucleotides

A 34-mer oligonucleotide (oligo) containing the ESX protein binding site (derived from HER2 gene promoter; TA5-oligo) and its complementary strand were synthesized from the Biopolymers facility (RPCI, Buffalo, NY) (sequence is shown in Fig. 1A). Oligos were gel-purified, annealed, and end-labeled with γ-^{32} P-[ATP] using T4-polynucleotide kinase (New England BioLabs, Beverly, MA) as described previously (10). ^{32}P-labeled 5' end-overhanging doubled-stranded oligo was used as the probe in gel mobility shift assays.

Mobility Shift Assay

Demonstration of proteins binding to their consensus binding sequences was performed by gel mobility shift assay. In general, proteins at the indicated concentrations and 1 nM ^{32}P-labeled oligo were incubated in a reaction buffer containing 25 mM Tris (pH 7.5), 30 mM KCl, 5% glycerol, 0.1% NP-40, bovine serum albumin (100 µg/ml), and 1 mM dithiothreitol. After incubation at room temperature for 30 min., samples were loaded onto 5% native polyacrylamide gels, running with TBE buffer (44.5 mM Tris-base, 44.5 mM boric acid, 1 mM EDTA, pH 8.3). Then, the gel was dried and exposed to Kodak film. The protein-DNA complex was quantitated using a computing laser densitometer (Molecular Dynamics, Sunnyvale, CA). The amount of protein which could complex with >90% of ^{32}P-labeled oligo was used for most experiments. Identification of protein-DNA complexes was confirmed by adding specific antibodies against proteins to the reaction.

Drug Assay

The ability of polyamides to interfere with the formation of the ESX-DNA complex was examined by a gel mobility shift assay. Assays were performed to

determine the ability of polyamides to inhibit ESX-DNA complex under equilibrium conditions. Experiments were set up to incubate polyamides with ^{32}P-labeled oligo at room temperature for 30 min. prior to the addition of ESX protein, or to complex ESX protein with the probe before adding polyamides. The inhibition of ESX-DNA complex formation by polyamides was measured by comparing drug-treated with non drug-treated samples. Investigation of the ability of polyamides to inhibit the AP-2-DNA complex was carried out in a similar manner. IC_{50} (concentration of drug required for 50% inhibition of protein-DNA complex formation) was used to express the activity of polyamides. Drug concentrations for IC_{50} were also expressed as r values (the molar ratio of drug to DNA base pairs).

In vitro Transcription.

In vitro transcription was performed in a buffer containing 12 mM Hepes-KOH (pH 7.9), 60 mM KCl, 7.5 mM $MgCl_2$, 12% glycerol, 0.12 mM EDTA, 0.12 mM EGTA, 1.2 mM DTT, and 0.6 mM PMSR. CsCl-purified plasmid DNA (RO6), composed of an insert DNA fragment from the HER2 promoter in the vector pCDNA3-Lue (Invitrogen, Carlsbad, CA), was linearized with restriction enzyme *Sph*I (New England BioLabs, Beverly, MA) and used as a DNA template. In a 25 µl reaction, 1 µg of *Sph*I-digested DNA, nuclear extracts, 0.5 µl of each nucleotide (20 mM of ATP, GTP, UTP, and 100 µM CTP), 10 µCi of α-^{32}P-[CTP] (800 Ci/mmole; NEN, Boston, MA), 1 µL of RNAsin (40 U/µL; Boerhringer Mannheim, Indianapolis, IN), and 1.4 µL of EDTA (2.5 mM) were incubated at 30 °C for 60 min. The reaction was stopped by adding 325 µL of 10 mM Tris-base (pH 8.0), 7 M urea, 350 mM NaCl, 1% SDS, and 100 µg tRNA, followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. Samples were resuspended in formamide-loading dye and heated at 90 ~ 95 °C for ≥ 1 min. before loading onto a 4%, 7 M urea-polyacrylamide gel. The ^{32}P signal from a dried gel was visualized using a Phosphorimager screen and quantitated with a computing laser densitometer (Molecular Dynamics, Sunnyvale, CA). The reaction was optimized by titration of nuclear extracts against 1 µg of DNA template in which a saturated condition was used for drug studies.

Effects of Polyamides on In Vitro Transcription

The ability of polyamides to inhibit *in vitro* transcription was analyzed in two ways. (i) DNA template was incubated with polyamides at the indicated concentrations in a total volume of 10 μL, for 30 min. prior to the addition of nuclear extracts and nucleotides. (ii) Pre-incubation of nuclear extracts and DNA template for 15 min. was followed by addition of polyamides for another 30 min. in a total volume of 10 μL and then nucleotides were added. All experiments were performed at 30 °C and the transcription reaction was allowed to proceed for 60 min. Percentage of inhibition of transcription was measured by comparing samples treated with polyamides to an untreated control. IC_{50} and r values were used to express the activity of polyamides. T3 transcript (250 bases; Promega Co., Madison, WI) was used as an internal control.

Time Course of Transcription in the Pressure of Drugs

The pattern of transcription from the HER2 promoter in the presence of PA-2E and distamycin was examined using a time course assay as described previously. Experimentally, DNA template incubated with SKBR-3 nuclear extracts for 15 min. at 30 °C followed by addition of drug (concentration used to inhibit about 30 ~ 50% of transcription) and the nucleotides (ATP, CTP, GTP, UTP), and the reaction was stopped at different time points (i.e., 5, 10, 30 and 60 min.). Transcripts from individual samples were normalized to an internal control and expressed as a percentage of relative transcription of an untreated control.

Gel Shift Experiments

Figure 13 shows the results of gel experiments results that demonstrate the binding of an oligonucleotide containing the ESX binding site of the HER2/neu promoter and the ESX protein in the presence of various concentrations of polyamide 2 ("PA-2E") or distamycin. Polyamide 2, ImPyPyPyPy-R2,4D-PyPyPyPyPy-β-Dp, blocked the binding of ESX protein to DNA in the concentration range of about 1 nM to about 100 nM. In contrast, distamycin blocked the binding of ESX protein to DNA in the concentration range of about 1 μM to about 10 μM.

PA-2E and distamycin target a similar portion (site 2) of the ESX DNA binding domain. A gel mobility shift assay was utilized to examine the activities of drugs on the ESX-DNA complex. Incubation of PA-2E and DNA followed by the addition of ESX resulted in a concentration-dependent inhibition of formation or the ESX-DNA complex. Ten nM PA-2E inhibited complex formation up to 95% while as little as 1 nM resulted in a detectable decrease on the complex (Figure 13A, lanes 2-4).

The pattern of inhibition of the ESX-DNA complex formation by distamycin is similar to that by PA-2E but significantly higher drug concentration is required. Distamycin at 200 nM diminished complex by about 95% (Figure 13B, lane 4). While PA-2E at 100 nM inhibited the ESX-DNA complex almost entirely, 100 nM distamycin had no effect on complex formation (Figure 3A, lane 2 and Figure 3B, lane 6). A quantitation of the data in Figure 16 indicated that 2.2 nM of PA-2E and 500 nM of distamycin are needed to inhibit complex formation by 50% (IC_{50}) (Table 1). The activity of individual drugs for inhibiting transcription factor-DNA complexes is also expressed as r values, the molar ratio of drug to DNA base pairs (Table 1).

Table 1. Drug Effects on Transcription Factor/DNA Complex Formation

		IC_{50}	
Drug	**Transcription Factor**	**[nM]**	**r value[1]**
PA-1E	ESX	5	0.16
	AP-2	48	1.55
PA-2E	ESX	2.2	0.07
	AP-2	>100	ND[2]
PA-3E	ESX	18	0.58
	AP-2	ND	ND
Distamycin	ESX	500	16.1
	AP-2	6000	193.5

[1] r value = the molar ratio of drug to DNA base pairs

[2] ND = not done

A quantitative comparison of the inhibition of the formation of the ESX/DNA complex is shown in Figure 14.

The inhibition of the ESX/DNA complex by polyamide 1 (ImPy-β-PyIm-R2,4D-PyPy-β-ImPy-β-Dp, "PA-1E") and polyamide 2 ("PA-2E") were presented either before exposure to ESX ("Before") or after the formation of the ESX/DNA complex ("After"). Little difference in the concentration producing 50% inhibition is seen with polyamide 1, although somewhat more inhibition was observed at lower concentrations if polyamide 1 was presented before the formation of the ESX/DNA complex (Figure 14A). In contrast, a concentration of about 2 nM polyamide 2 produced 50% inhibition if presented before the formation of the ESX/DNA complex, while a concentration of about 8 nM was needed to produce the same inhibition when applied after the formation of the ESX/DNA complex (Figure 14B).

The same relative effect on inhibition of *in vitro* transcription driven by the HER2/neu promoter was observed: polyamide 2 applied before (Figure 15B)was more effective than polyamide 1 (either before or after, Figure 15A) or polyamide 2 applied after (Figure 15B).

The ability of polyamides to inhibit the formation of ESX-DNA complex was measured under equilibrium conditions. However, for certain combinations of drugs and transcription factors, equilibrium requires greater concentrations of drug when drug is given to a preformed complex rather than prior to the transcription factor. In such cases, it is possible that the degree to which drugs compete to the transcription factor DNA binding domain is related to the time or concentration needed to establish equilibrium in regard to complex inhibition.

The data indicated that equal concentrations of PA-1E established an equilibrium under similar conditions for inhibiting complex formation whether drug was added before or after transcription factor complex formed (Figure 14 A). For example, at 10 nM drug concentration nearly the same level of complex was formed in both reactions within 30 minutes (Figure 14 A). In comparison, PA-2E required 10 fold more drug to inhibit complex formation under equilibrium conditions when drug was added after, compared to before, the complex formed (Figure 14B). To demonstrate that a longer incubation time

was required for PA-2E to reach equilibrium in comparison with PA-1E, a time course assay with 10 nM of PA-2E was performed adding drug following the formation of the transcription factor / DNA complex condition. The result indicated that the percentage of inihbition of ESX-DNA complex by PA-2E increased with longer incubation time and ≥4 hours was needed to reach equilibrium conditions (Figure 14 C).

The binding of AP-2 to the GC-rich sequences in the TA5-oligo was confirmed using gel mobility shift assays in the absence or presence of antibodies. AP-2 bound to the TA5 oligo and specific antibodies against AP-2 removed the protein-DNA complex, while non-specific antibodies or normal immunoglobulin had no effect on complex formation.

Since AP-2 interacts with GC-rich sequences which are a few base pairs upstream of the ESX binding site, it was of interest to know whether the polyamides that were designe for targeting the ESX binding site would affect the DNA binding of AP-2. Gel mobility shift assays were used, and Figure 17 A shows that PA-1E was cpable of inhibiting DNA binding of AP-2 in a concentration dependent manner with an IC_{50} of about 48 nM (Figure 17A, Table 1). In contrast, PA-2E was unable to block complex formation even at the highest drug concentration (100 nM) tested (Figure 17B – drug concentration greater than 100 nM caused smearing of the DNA under our assay conditions). The pattern of inhibition of the AP-2/DNA complex by distamycin was similar to that of PA-2E in that micromolar drug concentrations were required for both drugs to substantially inhibit AP-2/DNA complex formation (Figure 17C; Table 1). All the drugs were more efficient at inhibiting the complex formation of ESX than AP-2. PA-2E is the most specific inhibitor of ESX.

To determine whether the effects of polyamides onm transcription factor – DNA complex formation resulted in an ability to influence biological function, *in vitro* transcription assays were performed. The plasmid DNA(RO6) linearized with *Sph*I was used as a template and SKBR-3 nuclear extracts as transcription machinery, resulting in a ~760 base transcript. Drugs were incubated with DNA template prior to the addition of nuclear extracts and nucleotides. A representative gel demonstrated the ability of PA-2E to block synthesis of the 760 base transcript in a concentration-dependent manner. Five μM PA-2E inhibited transcript synthesis by 95% while 1 μM blocked the transcript less

than 50% compared with the untreated control. There was some evidence of the production of partial transcripts when higher drug concentrations were used. Inhibition of *in vitro* transcription by Pa-1E, PA-2E, Pa-3E and distamycin is shown in Figure 19. The order of potency at inhibiting transcription from the HER2/neu promoter differed from that for inhibiting transcription factor DNA complex formation: PA-2E > PA-3E > PA-1E > distamycin. The IC_{50} for individual drugs are listed in Table 2. Drugs concentrations of 1.4 μM for PA-2E, 2.4 μM for PA-3E , 3.2 μM for PA-1E and 7.4 μM for distamycin were required to inhibit transcription by 50%. Data are also expressed as r values so that the activities of individual drugs for their ability to inhibit transcription can be compared with inhibition of tanscription factor-DNA complexes.

Table 2. Drug Effects on *In Vitro* Transcription

	IC_{50}	
Drug	**[μM]**	**r value**
PA-1E	3.2	0.02
PA-2E	1.4	0.009
PA-3E	2.4	0.015
Distamycin	7.4	0.05

In Figure 18, the inhibition of the formation of the ESX/DNA complex by polyamide 1 ("PA-1E"), polyamide 2 ("PA-2E") or polyamide 3 (ImPy-β-PyPy-R2,4D-PyPy-β-PyPy-β-Dp, "PA-3E") is compared. Gel mobility shift assays were used to test the activity of the polyamides. PA-1E and PA-2E were similar in their ability to inhibit complex formation (Figure 18A). The IC_{50} for PA-1E was 5 nM compared to 2.2nM for PA-2E. In comparison, PA-3E was a relatively weak inhibitor of ESX-DNA complex and required a 9-fold higher drug concentration (18 nM) compared to PA-2E to inhibit 50% complex formation (Figure 18A, Table 1).

The relative effectiveness in inhibiting the formation of the ESX/DNA complex is consistent with the relative effectiveness in inhibition of *in vitro* transcription driven by the HER2/neu promoter. Figure 19 is a graphical representation of a quantitative comparison of the ability of polyamide 1 ("PA-1E"), polyamide 2 ("PA-2E"), polyamide 3 ("PA-3E") or distamycin ("Dist") to inhibit *in vitro* transcription driven by the HER2/neu promoter when the respective polyamides were presented before exposure to

ESX. The concentrations producing half-maximum inhibition were about 3 μM, about 1 μM, about 2.3 μM and about 7.3 μM, respectively.

* * *

All references cited herein are hereby incorporated by reference in their entireties, whether previously specifically incorporated or not.

The foregoing is intended to be illustrative of the present invention, but not limiting. Numerous variations and modifications of the present invention may be effected without departing from the true spirit and scope of the invention. While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth as follows in the scope of the appended claims.

What is claimed is:

1. A polyamide suitable for inhibiting the transcription of a gene, the polyamide comprising:

at least three complementary pairs of aromatic carboxamide residues, the complementary pairs of aromatic carboxamide residues being selected to correspond to a nucleotide sequence of an identified dsDNA target;

at least two aliphatic amino acid residues chosen from the group consisting of glycine, β-alanine, γ-aminobutyric acid, R 2,4-diaminobutyric acid, and 5-aminovaleric acid; and

at least one terminal alkylamino residue.

2. The polyamide of claim 1 wherein the complementary pairs of aromatic carboxamide residues selected to correspond to the nucleotide sequence of the identified dsDNA target are chosen from the group consisting of

Im/Py to correspond to the nucleotide pair G/C,

Py/Im to correspond to the nucleotide pair C/G,

Py/Py to correspond to the nucleotide pair A/T,

Py/Py to correspond to the nucleotide pair T/A,

Hp/Py to correspond to the nucleotide pair T/A, and

Py/Hp to correspond to the nucleotide pair A/T,

where Im is N-methyl imidazole, Py is N-methyl pyrrole, and Hp is 3-hydroxy N-methyl pyrrole.

3. The polyamide of claim 1 wherein the nucleotide sequence of the identified dsDNA target is chosen from the group consisting of

5'-TGCTTGA-3',

5'-AGAATGA-3',

5'-TGAGGAA-3',

5'-TGCTTGA-3',
5'-TGAGGAA-3',
5'-AGGAAGT-3',
5'-ATGAAGT-3',
5'-AGTATAA-3',
5'-AGTATAA-3',
5'-AGGAAGT-3',
5'-AGTATAA-3',
5'-AGTATAA-3',
5'-AACGGCT-3',
5'-TGCAGGCA-3',
5'-AACGGCT-3',
5'-TGCAGGCA-3', and
5'-AGGCAA-3'.

4. The polyamide of claim 1 wherein the transcription of the gene is inhibited by modulating the binding to dsDNA of a protein factor chosen from the group consisting of ESX, ETS, AP-2, and TBP.

5. The polyamide of claim 1 wherein at least one aliphatic amino acid residue is β-alanine.

6. The polyamide of claim 1 wherein the terminal alkylamino residue is a N,N-dimethylaminopropyl residue.

7. The polyamide of claim 5 wherein two β-alanine residues form complementary paired residues corresponding to a nucleotide pair chosen from the group A/T and T/A.

8. The polyamide of claim 1 wherein one aliphatic amino acid residue is R 2,4-diaminobutyric acid.

9. The polyamide of claim 1 having a binding affinity for the target dsDNA sequence of at least 10^9 M^{-1} and a selectivity of at least about two, selectivity being defined as the ratio of the binding affinity for the identified target dsDNA sequence to the binding affinity for a single base-pair mismatch dsDNA sequence.

10. A polyamide suitable for inhibiting the transcription of an oncogene, the polyamide comprising:

at least three complementary pairs of aromatic carboxamide residues, the complementary pairs of aromatic carboxamide residues being selected to correspond to a nucleotide sequence of an identified dsDNA target, at least two aliphatic amino acid residues chosen from the group consisting of glycine, β-alanine, γ-aminobutyric acid, R 2,4-diaminobutyric acid, and 5-aminovaleric acid, and at least one terminal alkylamino residue, the polyamide having a binding affinity at the target dsDNA sequence of at least 10^9 M^{-1} and a selectivity of at least about two, selectivity being defined as the ratio of the binding affinity for the nucleotide sequence of the identified dsDNA target to the binding affinity for a single base-pair mismatch sequence dsDNA.

11. The polyamide of claim 10 wherein the complementary pairs of aromatic carboxamide residues selected to correspond to the nucleotide sequence of an identified dsDNA target are chosen from the group consisting of

Im/Py to correspond to the nucleotide pair G/C,

Py/Im to correspond to the nucleotide pair C/G,

Py/Py to correspond to the nucleotide pair A/T,

Py/Py to correspond to the nucleotide pair T/A,

Hp/Py to correspond to the nucleotide pair T/A, and

Py/Hp to correspond to the nucleotide pair A/T,

where Im is N-methyl imidazole, Py is N-methyl pyrrole and Hp is 3-hydroxy N-methyl pyrrole.

12. The polyamide of claim 10 wherein the nucleotide sequence of the identified dsDNA target is chosen from the group consisting of

5'-TGCTTGA-3',

5'-AGAATGA-3',

5'-TGAGGAA-3',

5'-TGCTTGA-3',

5'-TGAGGAA-3',

5'-AGGAAGT-3',

5'-ATGAAGT-3',

5'-AGTATAA-3',

5'-AGTATAA-3',

5'-AGGAAGT-3',

5'-AGTATAA-3',

5'-AGTATAA-3',

5'-AACGGCT-3',

5'-TGCAGGCA-3',

5'-AACGGCT-3',

5'-TGCAGGCA-3', and

5'-AGGCAA-3'.

13. The polyamide of claim 10 wherein the transcription of the gene is inhibited by modulating the binding to dsDNA of a protein factor chosen from the group consisting of ESX, ETS, AP-2, and TBP.

14. The polyamide of claim 10 wherein at least one aliphatic amino acid residue is β-alanine.

15. The polyamide of claim 14 wherein two β-alanine residues form complementary paired residues corresponding to a nucleotide pair chosen from the group A/T and T/A.

16. The polyamide of claim 10 wherein one aliphatic amino acid residue is R 2,4-diaminobutyric acid.

17. The polyamide of claim 10 wherein the terminal alkylamino residue is a N,N-dimethylaminopropyl residue.

18. The polyamide of claim 10 wherein at least one Py of a carboxamide pair is replaced by a β-alanine.

19. A composition comprising a pharmaceutically acceptable excipient and a transcription-inhibiting amount of at least one polyamide, each polyamide comprising:

at least three complementary pairs of aromatic carboxamide residues, the complementary pairs of aromatic carboxamide residues being selected to correspond to a nucleotide sequence of an identified dsDNA target; and

at least two aliphatic amino acid residues chosen from the group consisting of glycine, β-alanine, γ-aminobutyric acid, R 2,4-diaminobutyric acid, and 5-aminovaleric acid, and at least one terminal alkylamino residue, the polyamide having a binding affinity at the target dsDNA sequence of at least 10^9 M^{-1} and a selectivity of at least about two, selectivity being defined as the ratio of the binding affinity for the identified target

dsDNA sequence to the binding affinity for a single base-pair mismatch dsDNA sequence.

20. The composition of claim 19 wherein the complementary pairs of aromatic carboxamide residues selected to correspond to the nucleotide sequence of the identified dsDNA target are chosen from the group consisting of

Im/Py to correspond to the nucleotide pair G/C,

Py/Im to correspond to the nucleotide pair C/G,

Py/Py to correspond to the nucleotide pair A/T,

Py/Py to correspond to the nucleotide pair T/A,

Hp/Py to correspond to the nucleotide pair T/A, and

Py/Hp to correspond to the nucleotide pair A/T,

where Im is N-methyl imidazole, Py is N-methyl pyrrole, and Hp is 3-hydroxy N-methyl pyrrole.

21. The composition of claim 19 wherein the nucleotide sequence of the identified dsDNA target is chosen from the group consisting of

5'-TGCTTGA-3',

5'-AGAATGA-3',

5'-TGAGGAA-3',

5'-TGCTTGA-3',

5'-TGAGGAA-3',

5'-AGGAAGT-3',

5'-ATGAAGT-3',

5'-AGTATAA-3',

5'-AGTATAA-3',

5'-AGGAAGT-3',

5'-AGTATAA-3',

5'-AGTATAA-3',
5'-AACGGCT-3',
5'-TGCAGGCA-3',
5'-AACGGCT-3',
5'-TGCAGGCA-3', and
5'-AGGCAA-3'.

22. The composition of claim 19 wherein at least one aliphatic amino acid residue is β-alanine.

23. The composition of claim 19 wherein the terminal alkylamino residue is a N,N-dimethylaminopropyl residue.

24. The composition of claim 23 wherein two β-alanine residues form complementary paired residues corresponding to a nucleotide pair chosen from the group A/T and T/A.

25. The composition of claim 19 wherein one aliphatic amino acid residue is R 2,4-diaminobutyric acid.

A

The HER-2/neu promoter

5'CCCGGGGGTCCTGGAAGCCACAAGGTAAACACAACACATCCCCCTCCTGGACTATGCAAT

TTTACTAGAGGATGTGGTGGGAAAACCATTATTTGATATTAAAACAAATAGGCTTGGGATGG

AGTAGGATGCAAGCTCCCCAGGAAAGTTTAAGATAAAACCTGAGACTTAAAAGGGTGTTAAG

AGTGGCAGCCTAGGGAATTTATCCCGGACTCCGGGGAGTCACCAGCCTCTGCATTTAGGGAT

AP-2

TCTCCGAGGAAAAGTGTGAGAACGGCTGCAGGCAACCCAGGCGTCCCGGCGCTAGGAGGGAC

GACCCAGGCCTGCGCGAAGAGAGGGAGAAAGTGAAGCTGGGAGTTGCCGACTCCCAGACTTC

GTTGGAATGCAGTTGGAGGGGGCGAGCTGGGAGCGCGCTTGCTCCCAATCACAGGAGAAGGA

ESX TATA

GGAGGTGGAGGAGGAGGGCTGCTTGAGGAAGTATAAGAATGAAGTTGTGAAGCTGAGATTCC

CCTCCATTGGGACCGGAGAAACCAGGGGAGCCCCCCGGGCAGCCGCGCGCCCCTTCCCACGG

GGCCCTTTACTGCGCCGCGCGCCCGGCCCCCACCCCTCGCAGCACCCCGCGCCCCGCGCCCT

CCCAGCCGGGTCCAGCCGGAGCCATGGGGCCGGAGCCGCAGTGAGCACCATG-3'

B

Schematic representation of the promoter

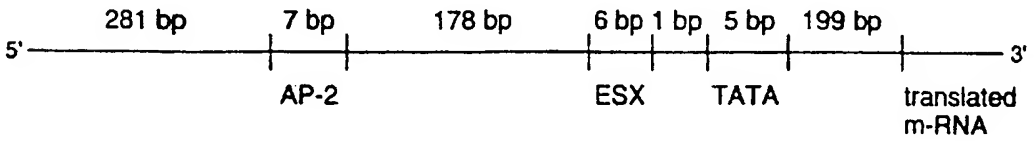
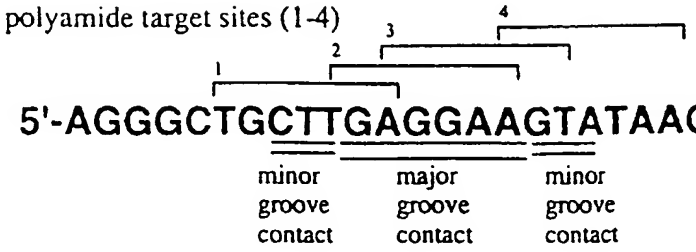


Fig. 1

The ESX promoter site



B

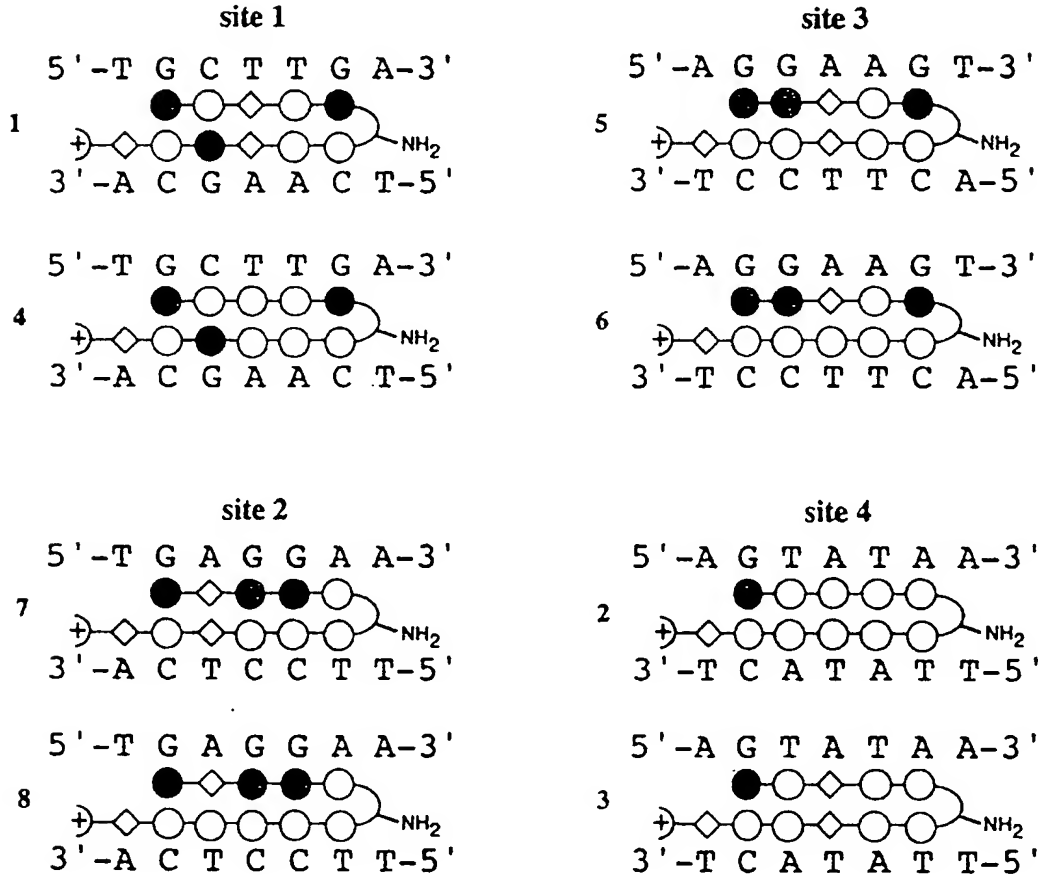
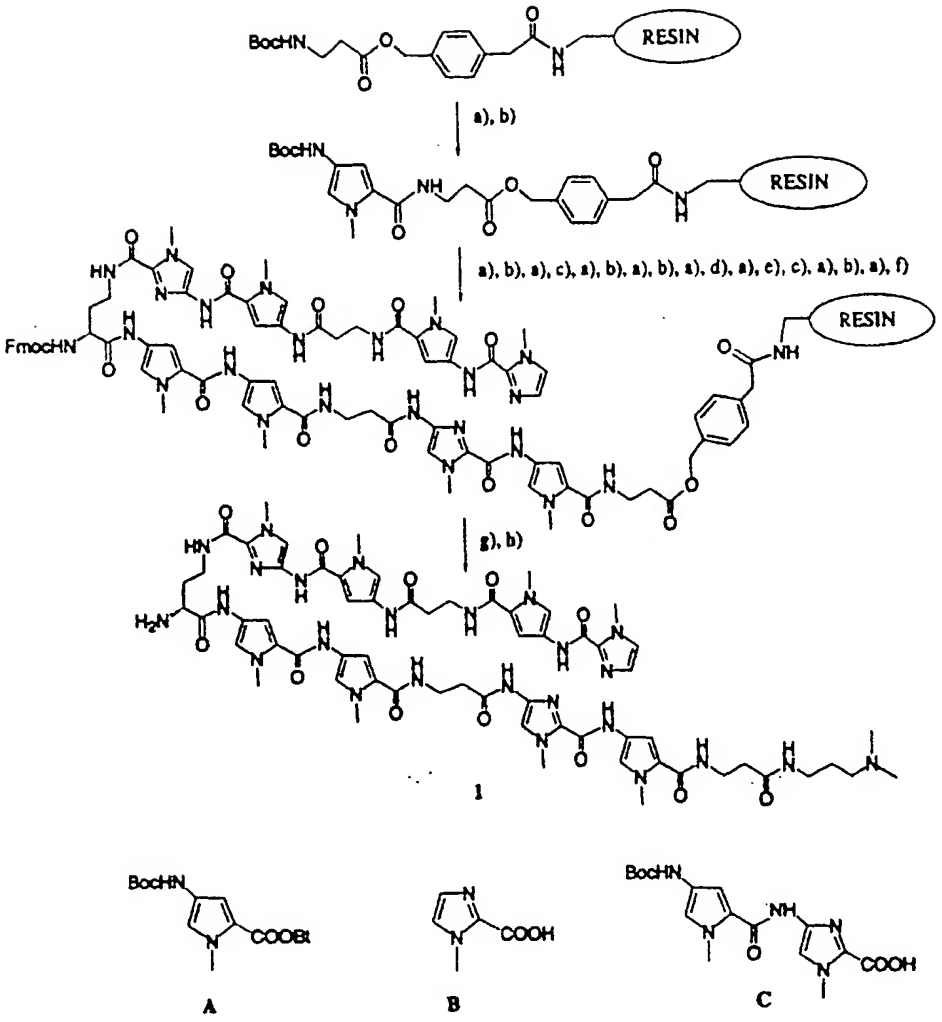
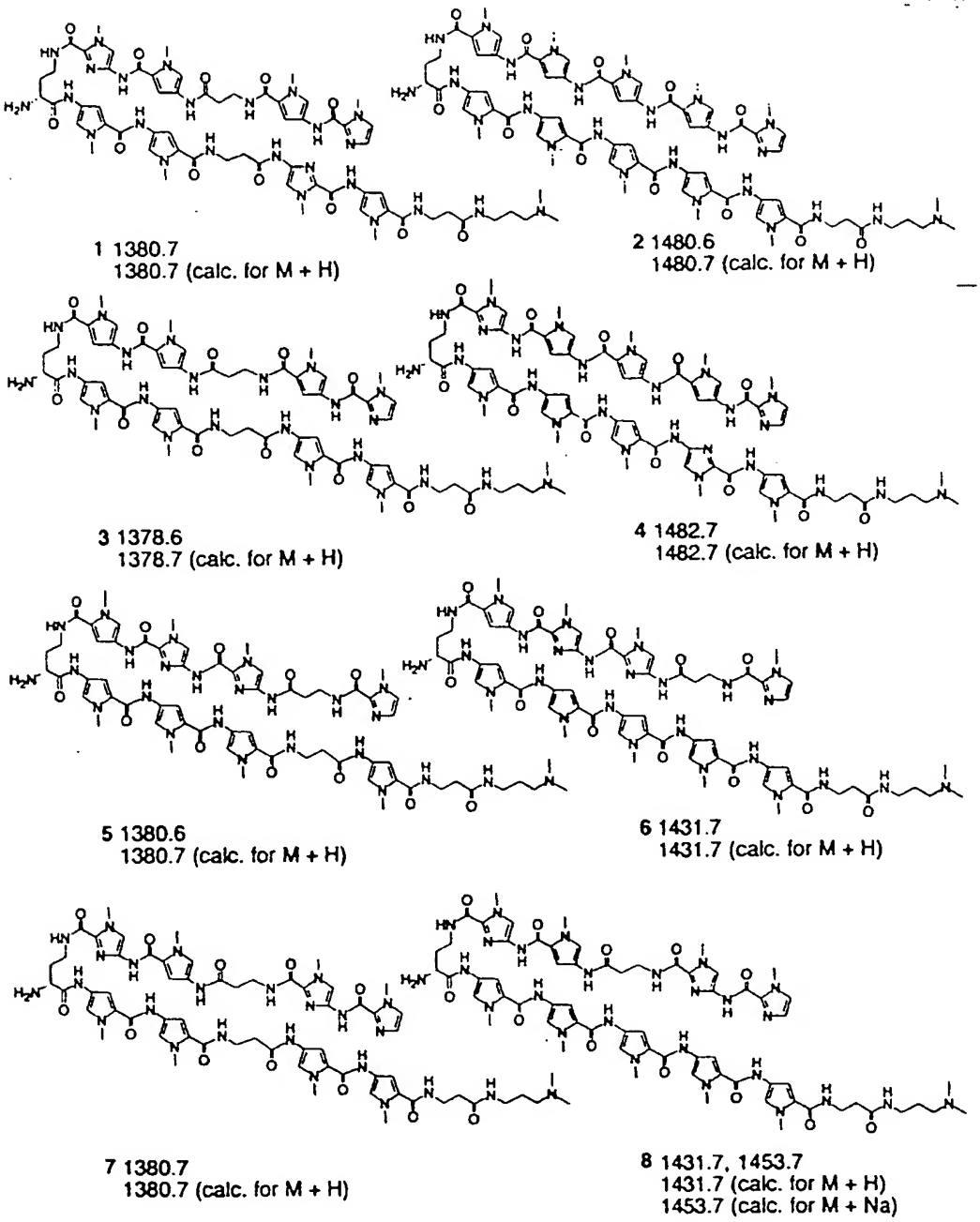


Fig. 2



a) CF_3COOH. b) A, $(iPr_2)NEt$, DMF. c) Boc-β-alanine, HBTU, $(iPr_2)NEt$, DMF. d) N-α-Fmoc-N-γ-Boc-D-diamino butyric acid, HBTU, $(iPr_2)NEt$, DMF. e) C, HBTU, $(iPr_2)NEt$, DMF. f) B, HBTU, $(iPr_2)NEt$, DMF. g) 20% piperidine, DMF. h) (N,N-dimethylamino)propylamine; prep. HPLC.

Fig. 3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1480.7 (calc. for M + H)
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1378.7 (calc. for M + H)
4 1482.7
1482.7 (calc. for M + H)
5 1380.6
1380.7 (calc. for M + H)
6 1431.7
1431.7 (calc. for M + H)
7 1380.7
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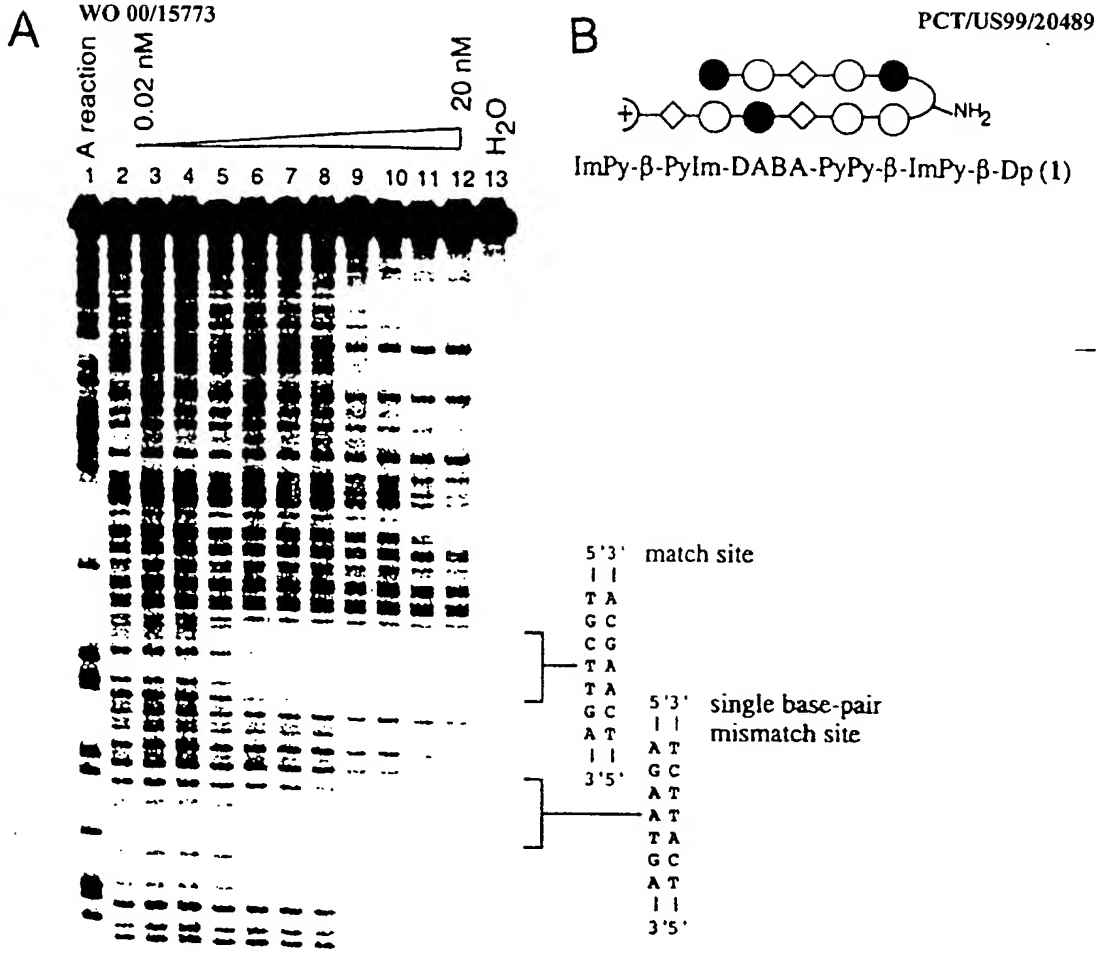
1453.7 (calc. for M + Na)

Fig. 4


A
A reaction
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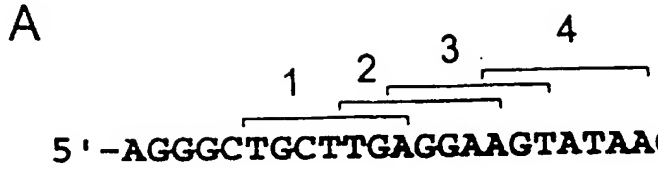
20 nM
H_2O
1 2 3 4 5 6 7 8 9 10 11 12 13
B
NH_2
ImPy-β-PyIm-DABA-PyPy-β-ImPy-β-Dp (1)
5'3' match site
single base-pair mismatch site

Fig. 5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1
2
3
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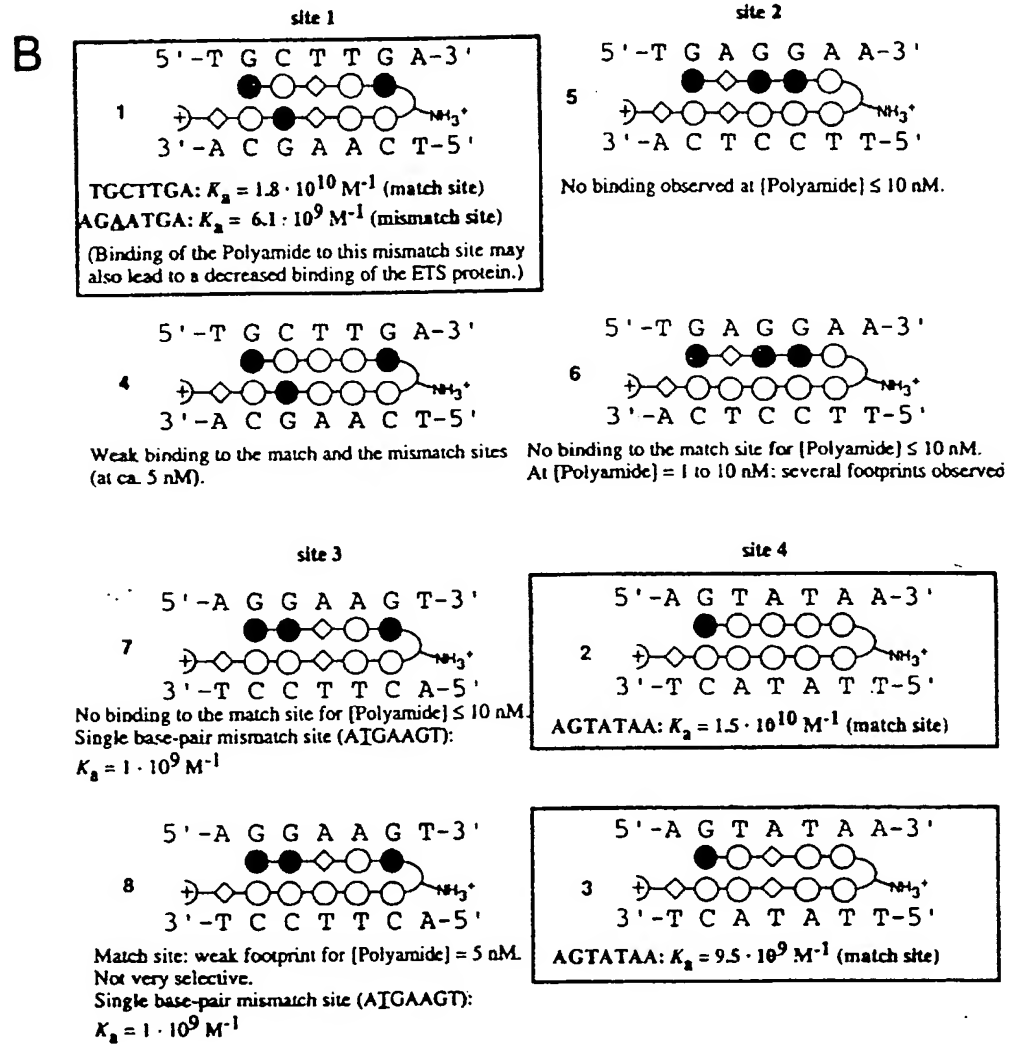
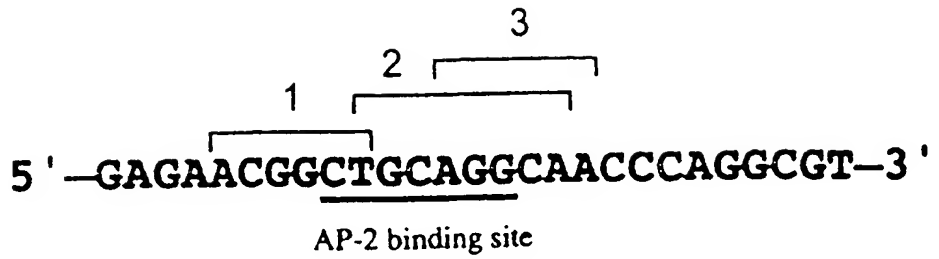

B
site 1
5'-T G C T T G A-3'
1
3'-A C G A A C T-5'
TGCTTGA: $K_a = 1.8 \cdot 10^{10}$ M^{-1} (match site)
AGAATGA: $K_a = 6.1 \cdot 10^9$ M^{-1} (mismatch site)
(Binding of the Polyamide to this mismatch site may also lead to a decreased binding of the ETS protein.)
site 2
5'-T G A G G A A-3'
5
3'-A C T C C T T-5'
No binding observed at [Polyamide] ≤ 10 nM.
5'-T G C T T G A-3'
4
3'-A C G A A C T-5'
Weak binding to the match and the mismatch sites (at ca. 5 nM).
5'-T G A G G A A-3'
6
3'-A C T C C T T-5'
No binding to the match site for [Polyamide] ≤ 10 nM.
At [Polyamide] = 1 to 10 nM: several footprints observed
site 3
5'-A G G A A G T-3'
7
3'-T C C T T C A-5'
No binding to the match site for [Polyamide] ≤ 10 nM.
Single base-pair mismatch site (ATGAAGT):
$K_a = 1 \cdot 10^9$ M^{-1}
site 4
5'-A G T A T A A-3'
2
3'-T C A T A T T-5'
AGTATAA: $K_a = 1.5 \cdot 10^{10}$ M^{-1} (match site)
5'-A G G A A G T-3'
8
3'-T C C T T C A-5'
Match site: weak footprint for [Polyamide] = 5 nM.
Not very selective.
Single base-pair mismatch site (ATGAAGT):
$K_a = 1 \cdot 10^9$ M^{-1}
5'-A G T A T A A-3'
3
3'-T C A T A T T-5'
AGTATAA: $K_a = 9.5 \cdot 10^9$ M^{-1} (match site)

Fig. 6

A


3
2
1
5'-GAGAACGGCTGCAGGCAACCCAGGCGT-3'
AP-2 binding site

B

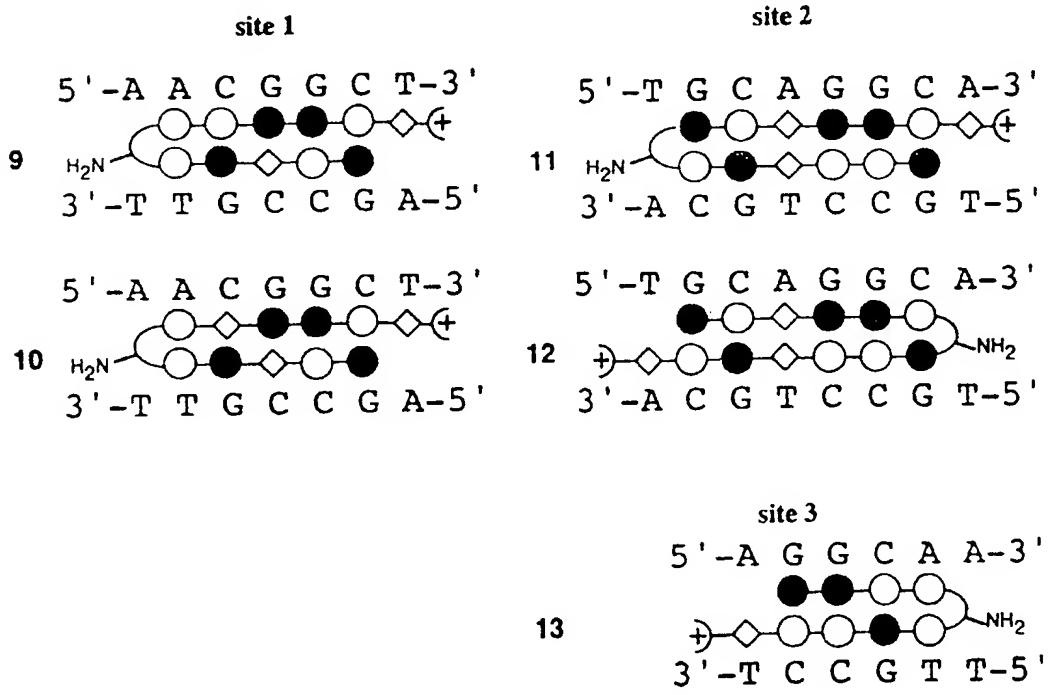
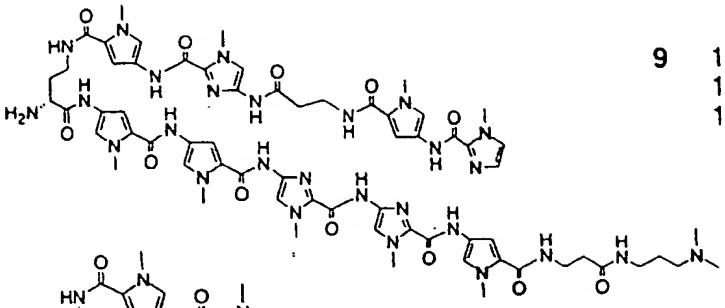
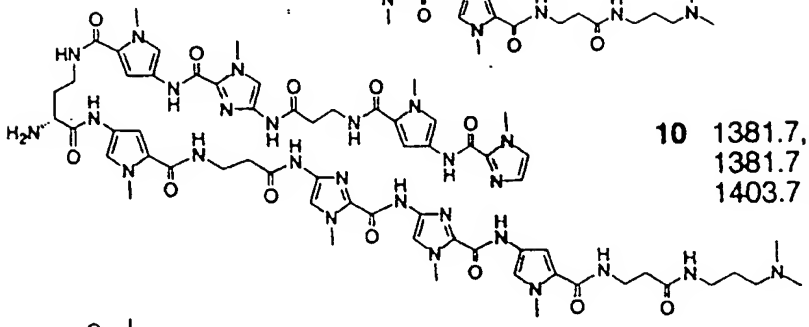

site 1
5'-A A C G G C T-3'
9
H2N
3'-T T G C C G A-5'
site 2
5'-T G C A G G C A-3'
11
H2N
3'-A C G T C C G T-5'
5'-A A C G G C T-3'
10
H2N
3'-T T G C C G A-5'
5'-T G C A G G C A-3'
12
NH2
3'-A C G T C C G T-5'
site 3
5'-A G G C A A-3'
13
NH2
3'-T C C G T T-5'

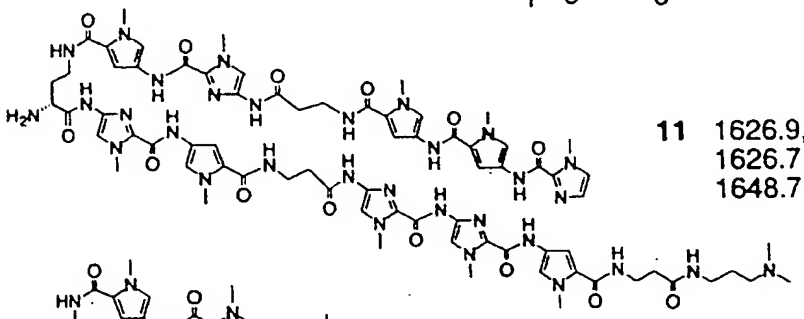
Fig. 7



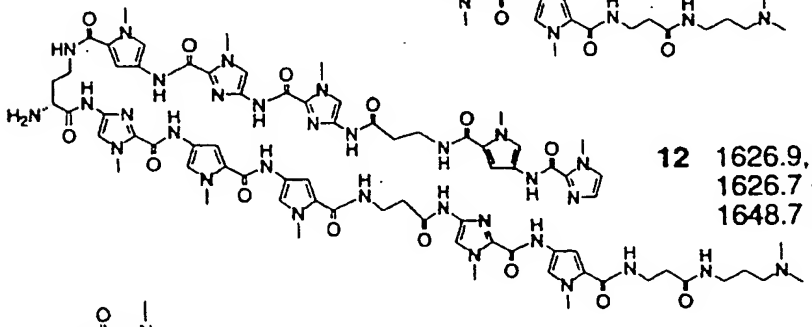
9 1432.8, 1454.8
1432.7 (calc. for M + H)
1454.7 (calc. for M + Na)



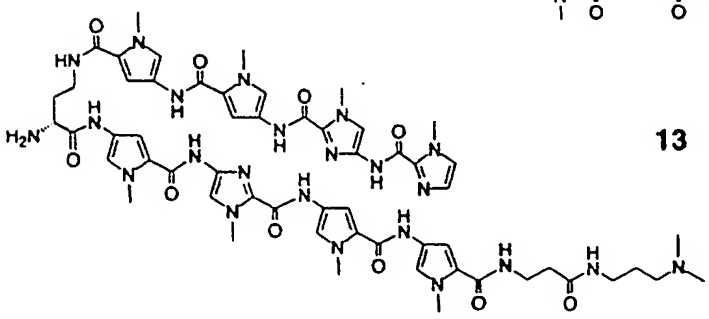
10 1381.7, 1403.6
1381.7 (calc. for M + H)
1403.7 (calc. for M + Na)



11 1626.9, 1648.9
1626.7 (calc. for M + H)
1648.7 (calc. for M + Na)



12 1626.9, 1648.6
1626.7 (calc. for M + H)
1648.7 (calc. for M + Na)



13 1238.6, 1260.6
1238.6 (calc. for M + H)
1260.6 (calc. for M +Na)

Fig. 8

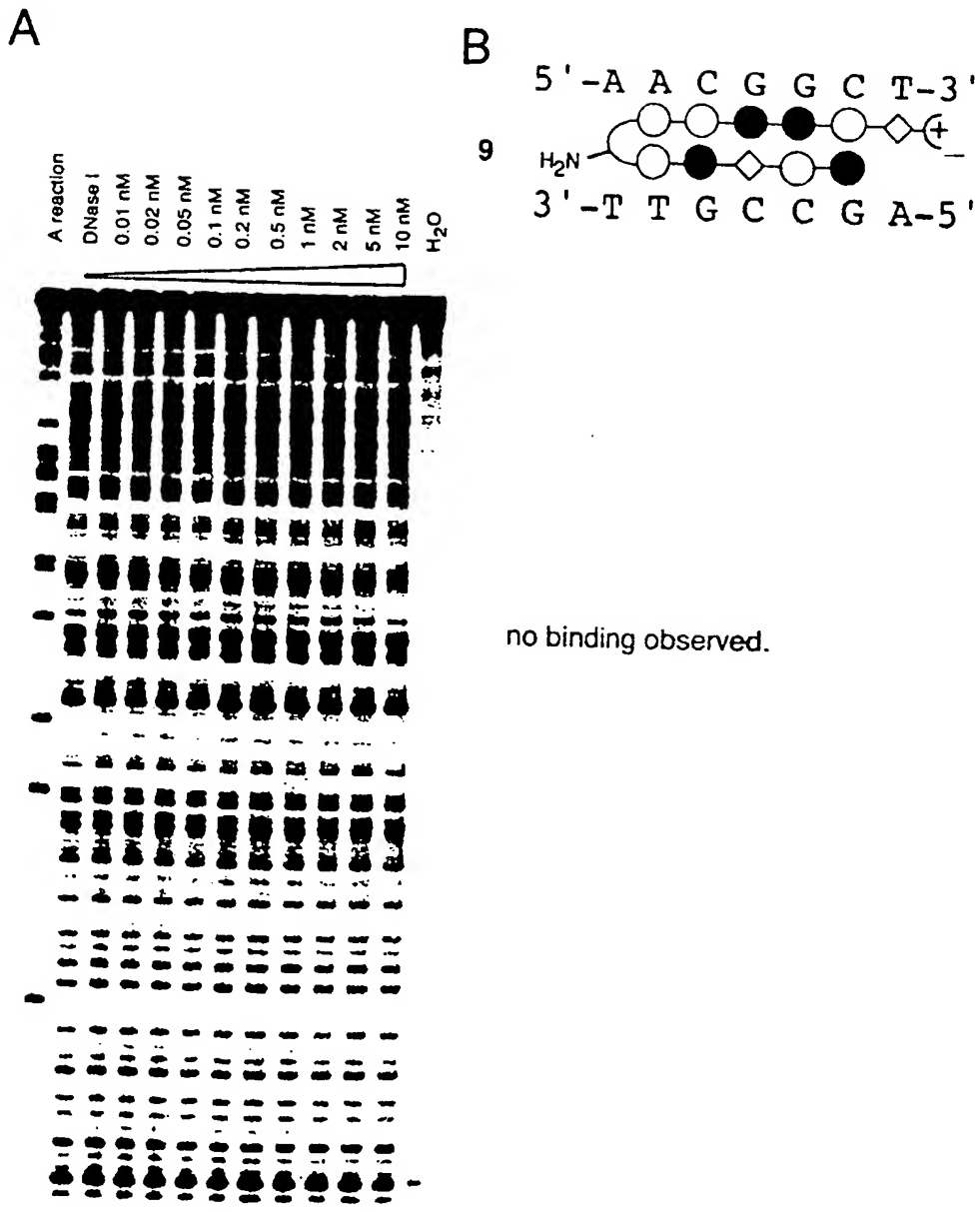

A
A reaction
DNase I
0.01 nM
0.02 nM
0.05 nM
0.1 nM
0.2 nM
0.5 nM
1 nM
2 nM
5 nM
10 nM
H_2O
B
5'-A A C G G C T-3'
9
H_2N
3'-T T G C C G A-5'
no binding observed.

Fig. 9

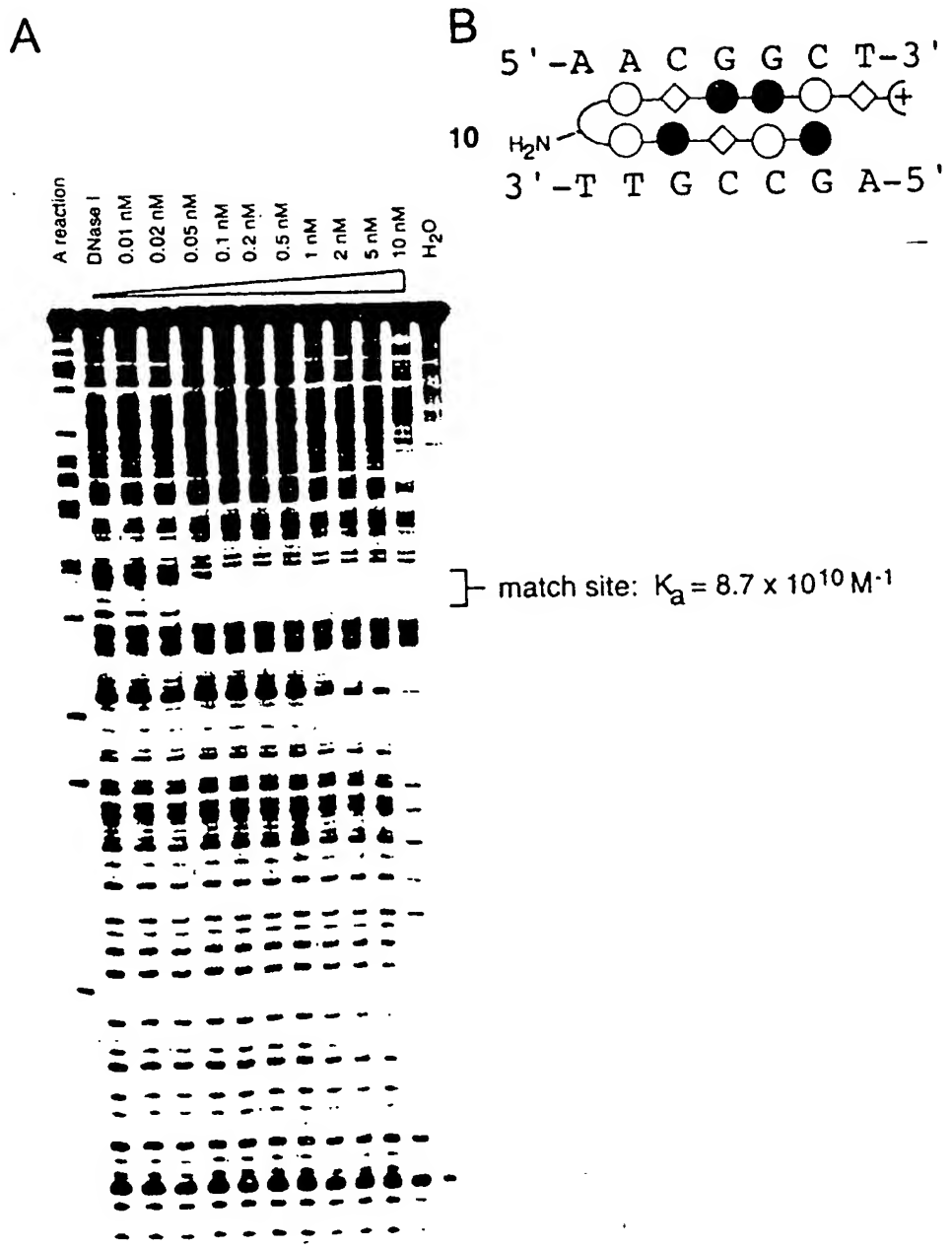

A
B
5'-A A C G G C T-3'
10
H_2N
3'-T T G C C G A-5'
A reaction
DNase I
0.01 nM
0.02 nM
0.05 nM
0.1 nM
0.2 nM
0.5 nM
1 nM
2 nM
5 nM
10 nM
H_2O
match site: $K_a = 8.7 \times 10^{10}\ M^{-1}$

Fig. 10

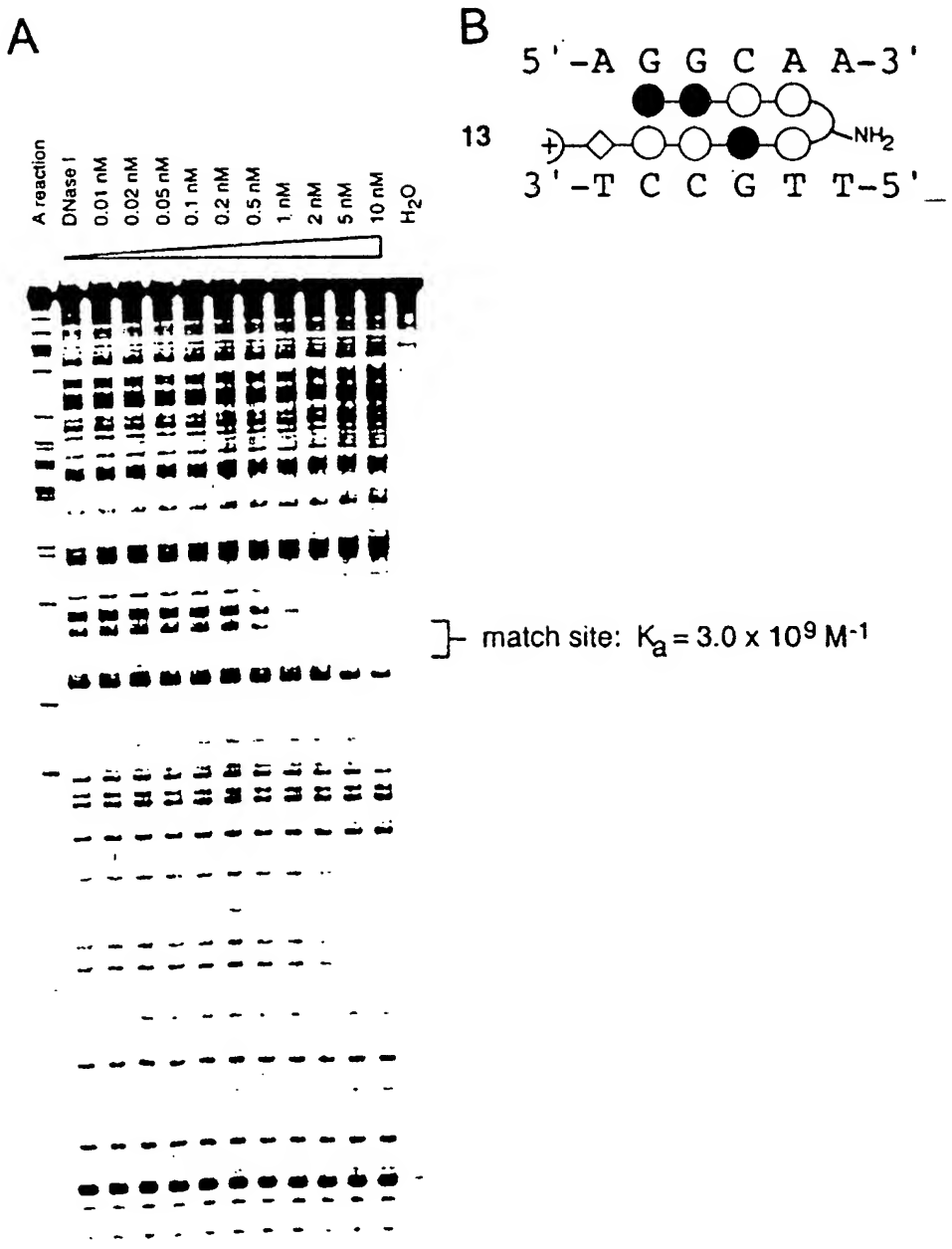
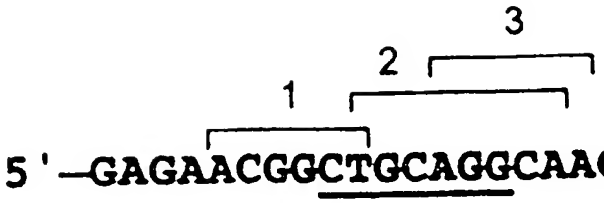

A
A reaction
DNase I
0.01 nM
0.02 nM
0.05 nM
0.1 nM
0.2 nM
0.5 nM
1 nM
2 nM
5 nM
10 nM
H2O
B
5'-A G G C A A-3'
13
NH2
3'-T C C G T T-5'
match site: Ka = 3.0 x 10^9 M^-1

Fig. 11

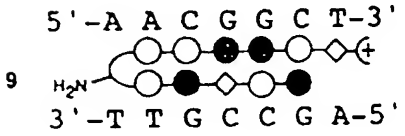
A



AP-2 binding site

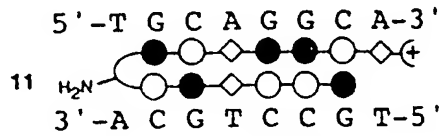
B

site 1

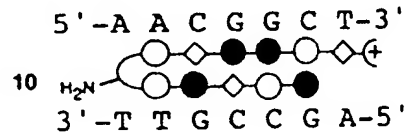


No binding observed at concentrations of $9 < 10$ nM.

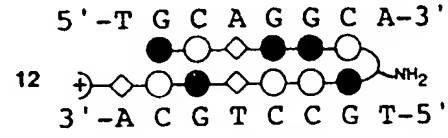
site 2



TGCAGGCA: $K_a = 6.5 \cdot 10^9\ M^{-1}$ (match site)
(Several binding sites with K_a = ca. $2 \cdot 10^8\ M^{-1}$.)

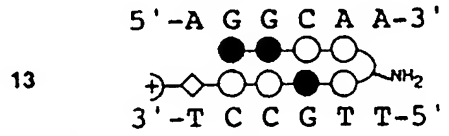


AACGGCT: $K_a = 8.7 \cdot 10^{10}\ M^{-1}$ (match site)
(Very selective!)



TGCAGGCA: $K_a = 5.8 \cdot 10^9\ M^{-1}$ (match site)
(Several binding sites with K_a = ca. $2 \cdot 10^8\ M^{-1}$.)

site 3



AGGCAA: $K_a = 3.0 \cdot 10^9\ M^{-1}$ (match site)
No other binding sites observed at concentrations of $13 < 10$ nM.

Fig. 12

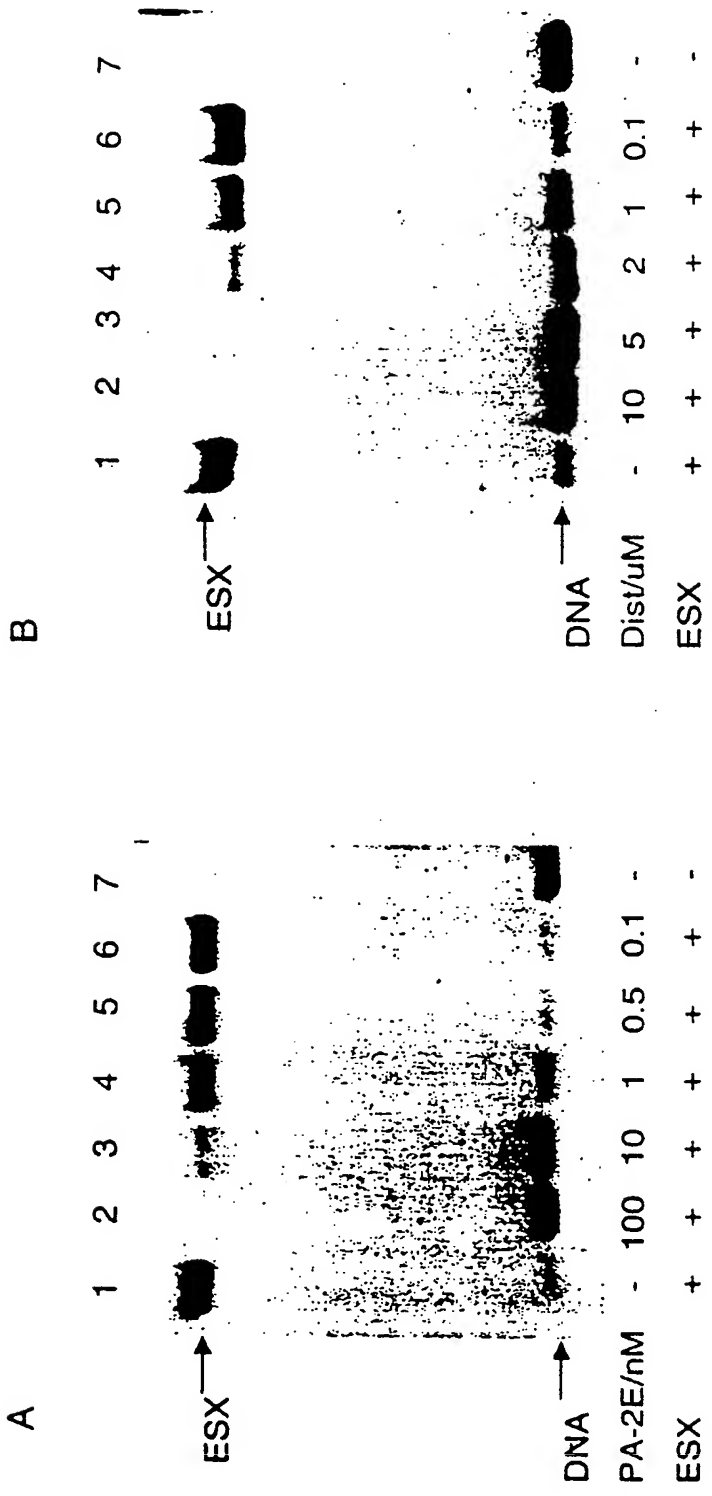

A
1 2 3 4 5 6 7
ESX
DNA
PA-2E/nM - 100 10 1 0.5 0.1 -
ESX + + + + + + -
B
1 2 3 4 5 6 7
ESX
DNA
Dist/uM - 10 5 2 1 0.1 -
ESX + + + + + + -

Fig. 13

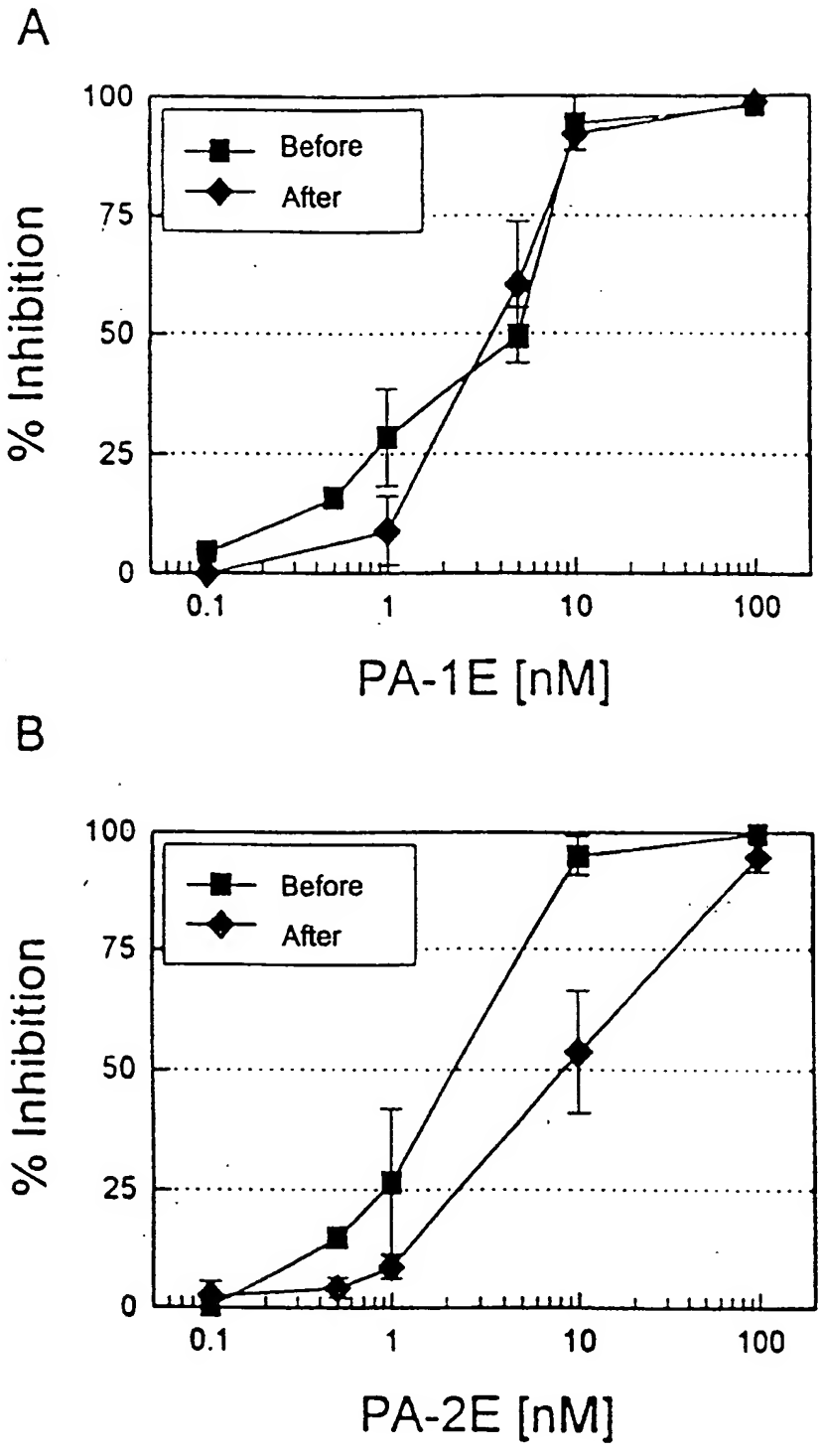

A
100
75
50
25
0
% Inhibition
Before
After
0.1
1
10
100
PA-1E [nM]
B
100
75
50
25
0
% Inhibition
Before
After
0.1
1
10
100
PA-2E [nM]

Fig. 14

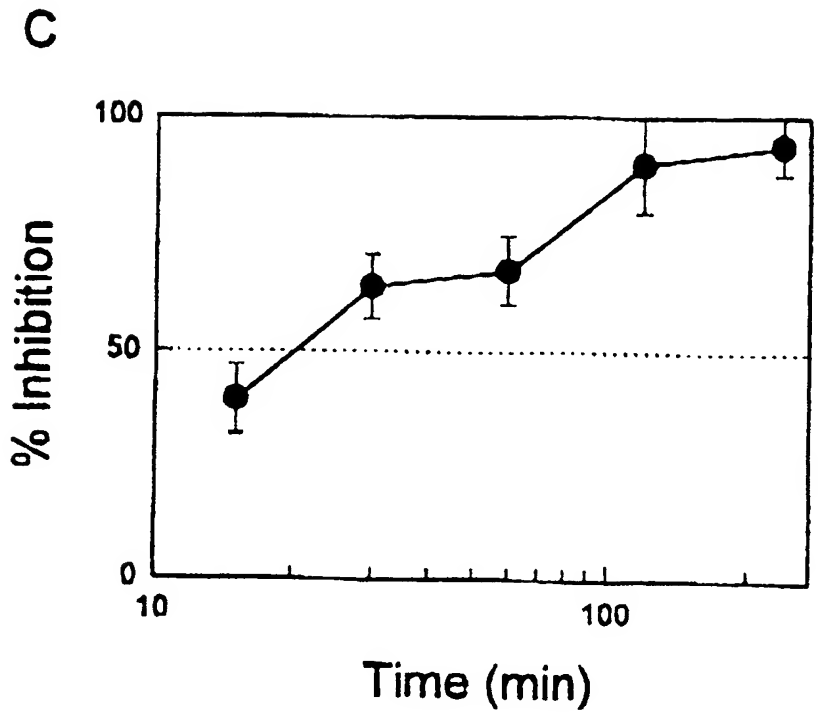


Fig. 14

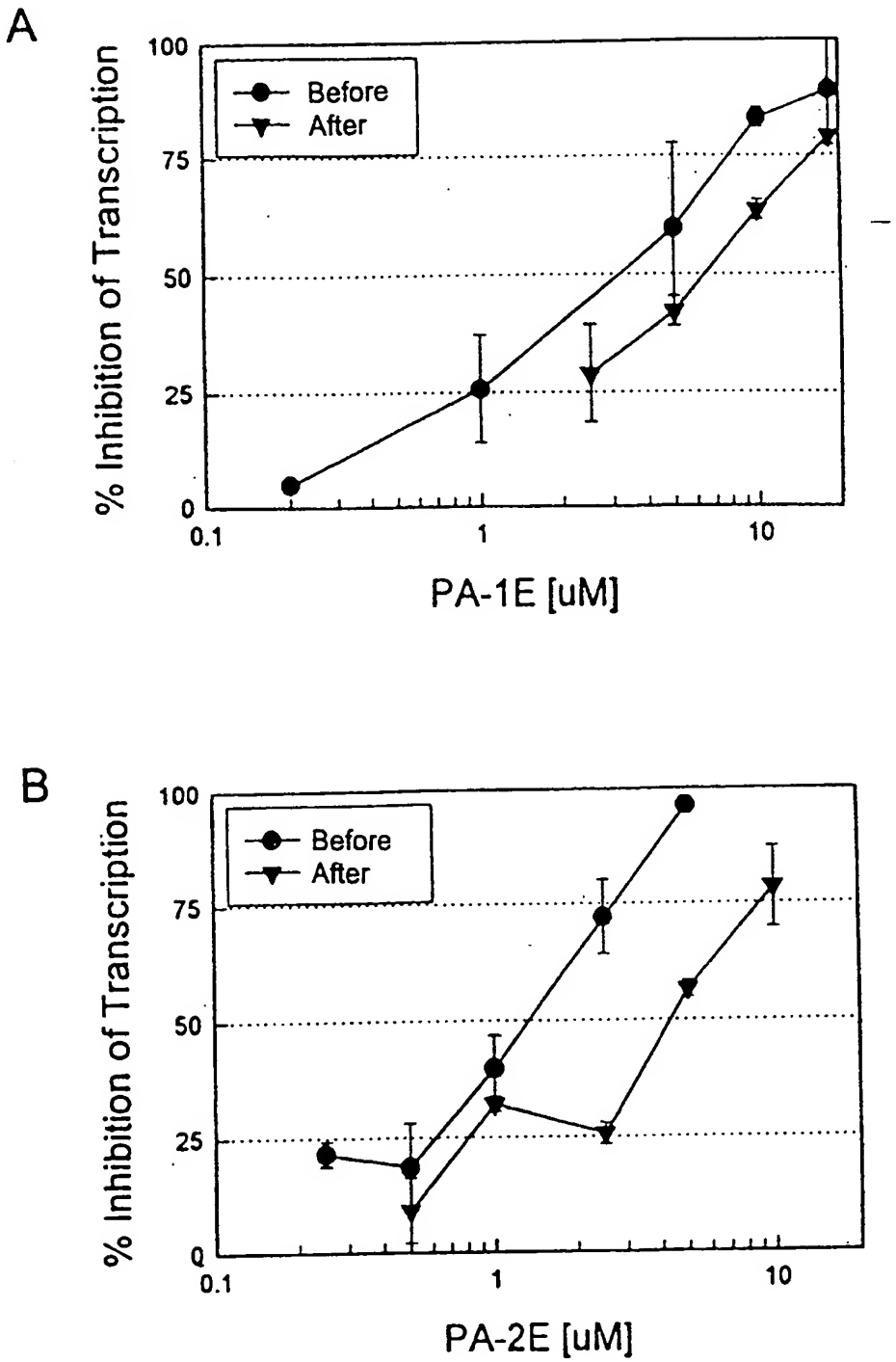

A
% Inhibition of Transcription
100
75
50
25
0
Before
After
0.1
1
10
PA-1E [uM]
B
% Inhibition of Transcription
100
75
50
25
0
Before
After
0.1
1
10
PA-2E [uM]

Fig. 15

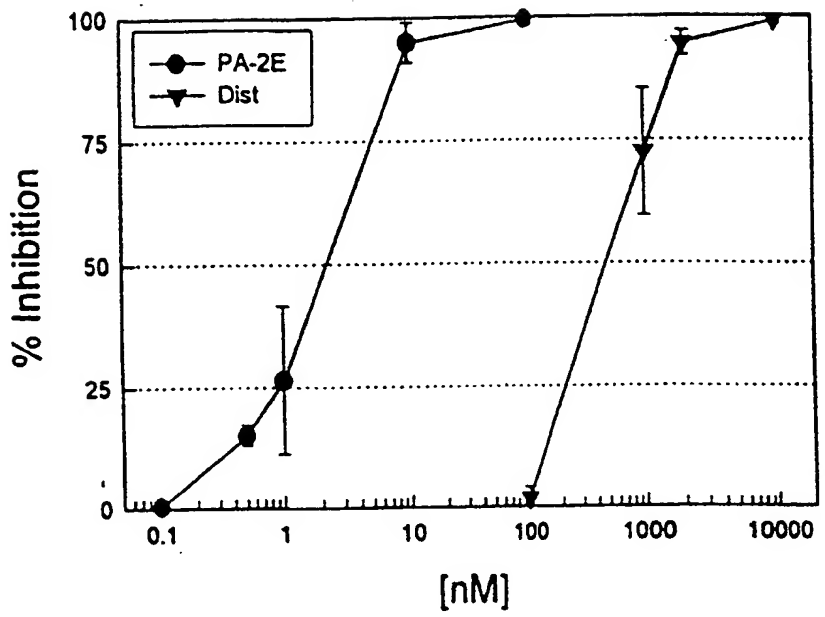


Fig. 16

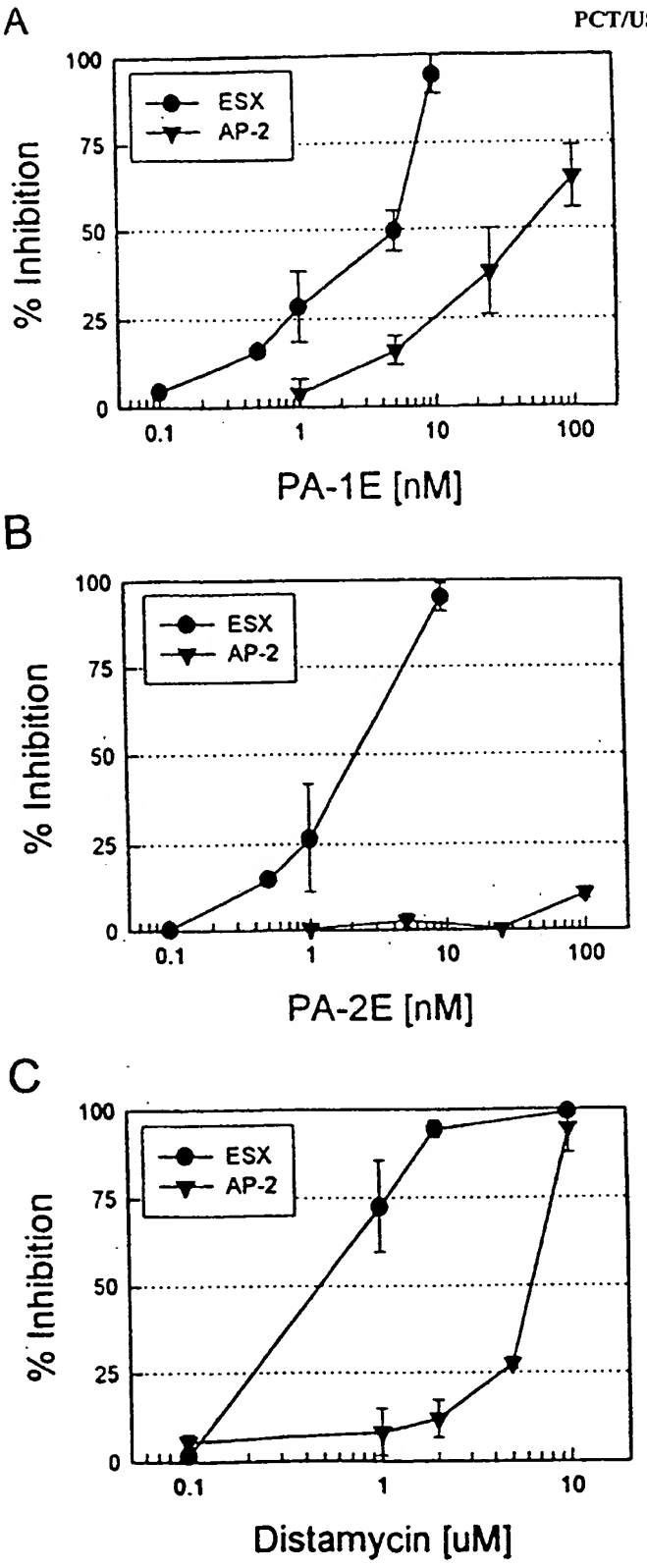
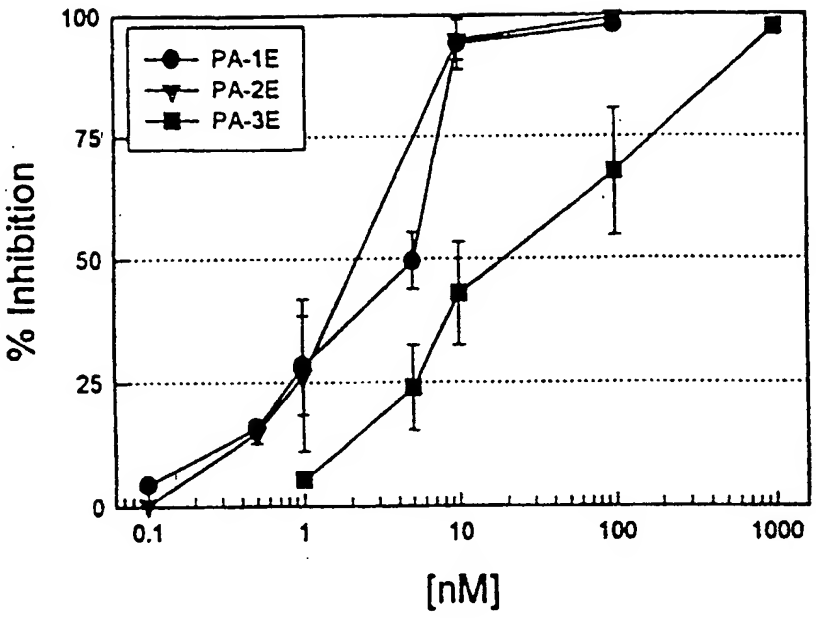
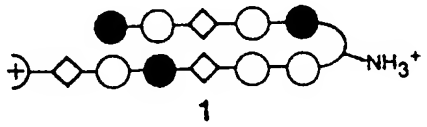

A
100
75
50
25
0
% Inhibition
ESX
AP-2
0.1
1
10
100
PA-1E [nM]
B
100
75
50
25
0
% Inhibition
ESX
AP-2
0.1
1
10
100
PA-2E [nM]
C
100
75
50
25
0
% Inhibition
ESX
AP-2
0.1
1
10
Distamycin [uM]

Fig. 17

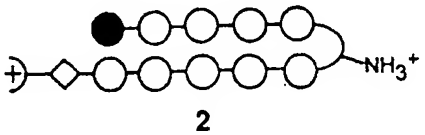
A


100
75
50
25
0
% Inhibition
PA-1E
PA-2E
PA-3E
0.1
1
10
100
1000
[nM]

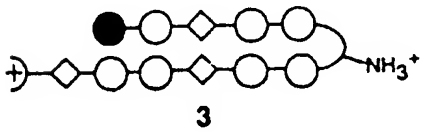
B


NH3+

1


NH3+

2


NH3+

3

Fig. 18

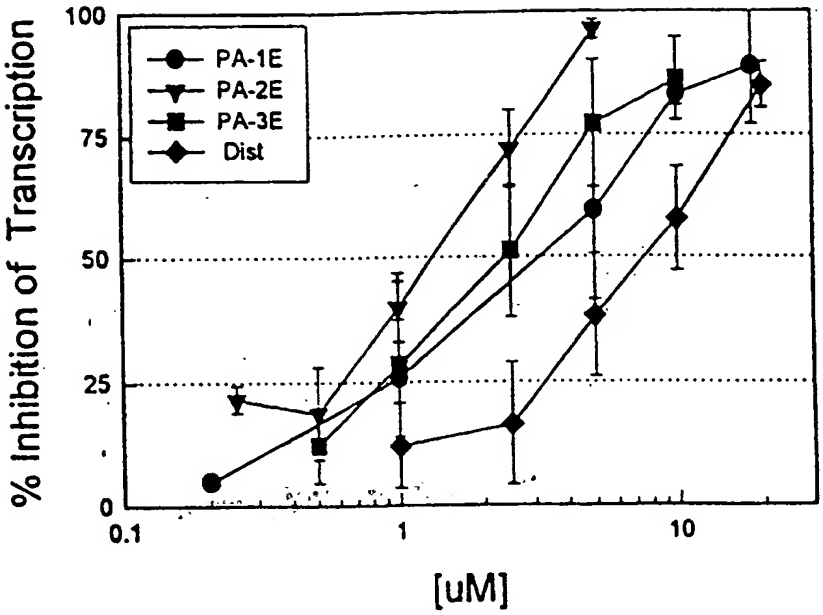

% Inhibition of Transcription
100
75
50
25
0
0.1
1
10
[uM]
PA-1E
PA-2E
PA-3E
Dist

Fig. 19